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In vitro modulation of the microglial phenotype by physiological and pathological stimuli: perspectives for neuroinflammatory and neurodegenerative diseases

Coordinator: Prof. **Daniela PAROLARO**

Director of studies: Dr. **Daniele ZACCHETTI**

Tesi di DOTTORATO di RICERCA di:

Alessandra CONSONNI

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1. INTRODUCTION

1.1 "Neuroinflammation" and its relevance in neurodegeneration

The term neuroinflammation is used to describe the complex integration of responses within the Central Nervous System (CNS) as a result of inflammatory processes occurring in several neurological disorders, with particular regards to neurodegenerative diseases (OCallaghan, 2008).

The CNS is recognized as an immune privileged site in which immune responses are tightly regulated (Carson, 2006). It is anatomically and physiologically protected by the presence of the Blood Brain Barrier (BBB) and the Cerebro-Spinal Fluid (CSF), which control the passage of ions, macromolecules and immune cells, thus maintaining the homeostasis required for the neuronal functions and the host defense. Moreover, other mechanisms have been proposed to contribute to CNS immune privilege. First of all CNS parenchyma lacks dendritic cells and lymphatic vessels (reviewed by Perry, 1998); secondly, there is a local production of anti-inflammatory mediators and constitutive expression of Fas ligand, responsible for Fas-mediated killing of CNS-infiltrating immune cells (Bechmann, 1999; Flugel, 2000).

Despite the immune-privileged context, CNS is constantly surveyed by the immune system and innate inflammatory responses can occur (Amor, 2010). The presence of dendritic cells and macrophages has been described in strategic locations in the meninges and choroid plexuses, to shield the ventricular/sub arachnoid compartment (McMenamin, 1999). Moreover, perivascular macrophages surround small and medium size cerebral vessels and display endocytic, phagocytic and immune regulatory functions, thus ensuring protection at the BBB level (Thomas, 1999; Williams, 2001). Therefore, a well-elaborated network of innate immune cells seems to guard all the entries into the CNS parenchyma.

A role for immune responses that involves antigen presentation and release of pro-inflammatory molecules in neurodegenerative diseases had been described for a decade before the term neuroinflammation came into use (McGeer, 1987; Griffin, 1989). Neuroscientists spoke of "reactive gliosis" in describing endogenous CNS tissue responses to injury. This term referred to the accumulation of enlarged glial cells,

appearing immediately after CNS injury has occurred. These new studies led to the recognition that glia, especially microglial cells, trigger a response to tissue insult, resulting in a complex pattern of actions and promoting the release of pro-inflammatory mediators. Microglia are now identified as the leading component of an intrinsic brain innate immune system, upon which adaptive immunity is built (Medzhitov, 2000). However, the inflammatory scenario in the CNS is very complex: microglia, astrocytes and endothelial cells may act as antigen presenting cells, and neurons can promote immune activation through the release of complement factors, chemokines, danger-associated molecular patterns (DAMPs), and matrix metalloproteinase (MMPs) enzymes.

While in most of the peripheral tissues, activation of immune cells triggers leukocyte infiltration, this is rare in brain, unless there has been a breakdown of the BBB (Sroga, 2003). In acute reactions to injury, even when BBB is intact, neuronal insults trigger the activation of glia, which secretes cytokines and acquires a phagocytic phenotype, in order to regenerate the physiological homeostasis (Kreutzberg, 1996). Although these responses might be included in the process known as “neuroinflammation”, with this term we also refer to more chronic conditions in which the cumulative ill effects of immunological glia activation contribute to and expand the initial pathogenic insult, thus sustaining or worsening the disease process.

The concept of chronic inflammation is more relevant in the context of CNS pathologies, as opposed to CNS injury. Chronic microglial activation is now recognized as an important component of neurodegenerative diseases, and this neuroinflammatory factor contributes to neuronal dysfunction, injury, and loss, i.e. to disease progression.

The recognition of microglia as the intrinsic immune system of the brain has led to a more modern concept of neuroinflammation. This vision of microglia-driven neuroinflammatory responses, with neuropathological consequences, has extended the older vision of passive glial responses that are inherent in the concept of "reactive gliosis".

1.2 Overview of glial cells

For very long time, the non-neuronal cells of the CNS have been considered secondary elements having only a passive role in the support of neuronal network. In fact, glial cells, sometimes called neuroglia or simply glia, were initially defined by Rudolf Virchow in 1858 as the connective tissue that binds nervous elements together. Soon after, in 1870s, the cellular nature of glia was firmly established by Camillo Golgi (Golgi, 1873).

Neuroglia is classified in two major classes: macroglia and microglia (Ling, 1973). Macroglial cells include oligodendrocytes, which coat axons with myelin, and astrocytes, the most abundant and heterogeneous population of glial cells. Microglia make up the innate immune system of the CNS and are key cellular mediators of neuroinflammatory processes. Their involvement in neurodegenerative diseases is highly debated. However, microglial cell activation is generally thought to be very important in the neurodegenerative processes (Streit, 2004).

Astrocytes, the most abundant glial cells in brain, were named by Michael Von Lenhossek (Lenhossek, 1891). Subsequently, Rudolf Albert von Kolliker and William Lloyd Andriezen (Kolliker, 1889; Andriezen, 1893) sub-classified them into protoplasmic and fibrous astrocytes, located in the grey and white matter, respectively. Santiago Ramon y Cajal further studied astrocytes and their interactions with other neural elements (Ramon y Cajal, 1911).

As the Greek name implies, glia were originally referred to as the glue of the nervous system. Ever since, this original concept has been continuously re-evaluating, as new functions emerged, thus revealing the complexity of neuron-glia interaction (Pfrieger, 1997). In particular, glial cells are now known to be critical players in every major aspect of brain development, function, and disease (Barres, 2008), indicating their important active role in the physiology of the nervous system.

Glial functions start during early brain development when they support neuronal migration, survival and differentiation, by producing molecules able to modify the growth of axons and dendrites; moreover, they contribute to: formation of the blood brain barrier, organization of the myelin sheath, trophic and metabolic support to neurons, synaptic transmission, as well as clearance of cell debris after neuronal injury

or death (Zhang, 2003; Volterra and Meldolesi 2005).

1.2.1 Glial cells and the microarchitecture of the brain

As mentioned before, the brain is separated from the rest of the body by the presence of the BBB, which contributes to the homeostatic equilibrium of the nervous system.

The brain tissue shows a high degree of hierarchical organization, with anatomical segregation of different types of cells endowed with specific functions. Glial cells are now considered to be the basic elemental structures of the brain microarchitecture that divide parenchyma into morphologically independent structural domains (Bushong, 2004).

In the grey matter, astrocytes share tissue territories by creating micro-anatomical domains within the extension of their processes; microglia surveil the brain by forming non-overlapping ‘‘defense’’ domains, while oligodendrocytes form anatomically segregated nodes of Ranvier on the axons enwrapped by their processes.

Within their anatomical domains, astroglial membranes cover synapses and establish contacts with neuronal membranes, as well as with blood vessels. Moreover, astrocytes are further integrated into astroglial syncytia through gap junctions localized on the peripheral processes where astrocytic domains overlap. Gap junctions formed by intercellular channels, the connexins, form pathways for intercellular diffusion of many molecules, employed for long-range signalling (Giaume, 1998). Importantly, astroglial syncytia are formed within defined anatomical structures, further contributing to the hierarchical organization of the brain.

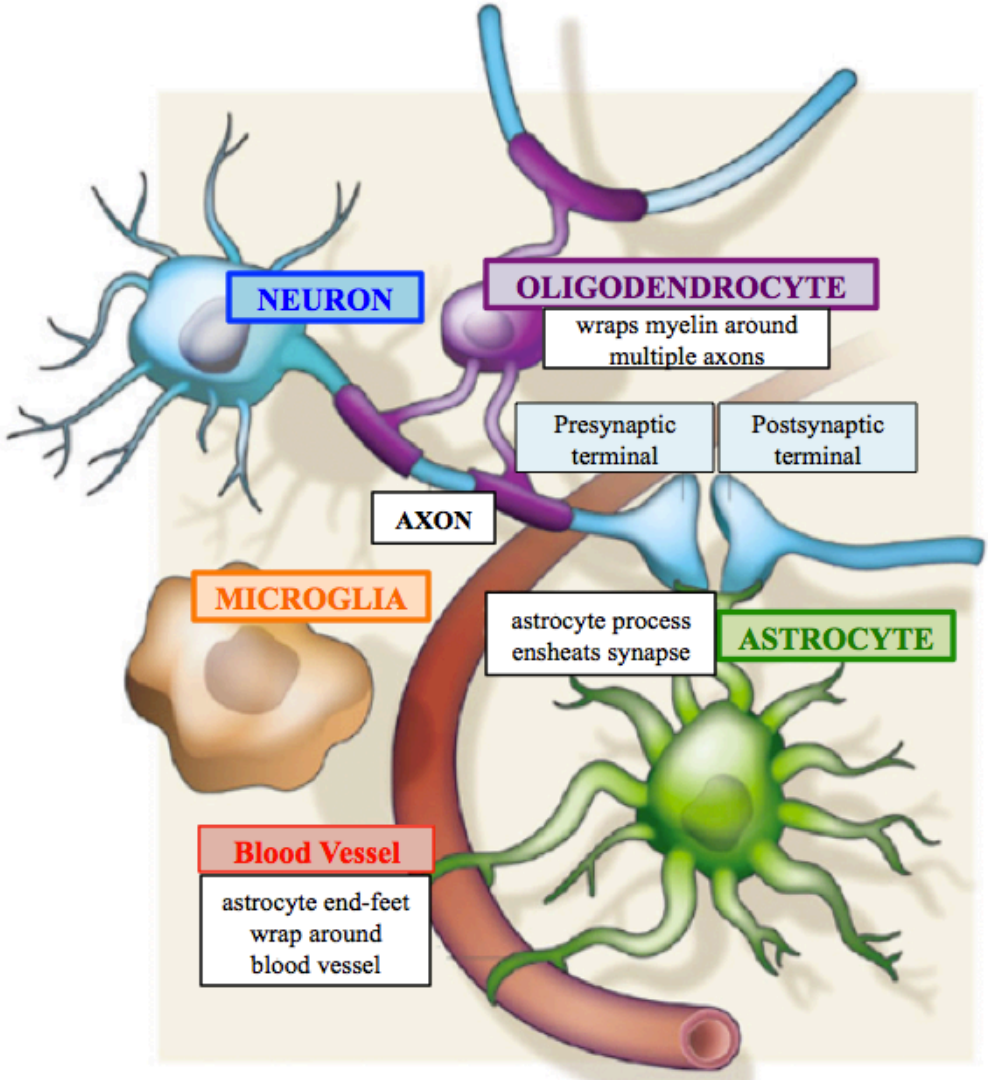


Fig. 1.1: Glial cells interactions and functions

Different types of glial cells interact with neurons and the surrounding blood vessels. Oligodendrocytes wrap myelin around axons to speed up neuronal transmission. Astrocytes extend processes that ensheath blood vessels and synapses. Microglia keep the brain under surveillance for damage or infection (modified from Allen and Barres, 2009)

1.2.2 Astrocytes: role and functions

Astrocytes are the most abundant, morphologically heterogeneous and functionally diverse neuroglia cells. They are generically called 'astroglia', a cell class that covers all non-myelinating macroglial cells in the CNS.

Morphologically astrocytes are divided into two types, protoplasmic and fibrous (Miller, 1984). Protoplasmic astrocytes are found in the grey matter extending long processes, with which they contact neurons, and end-feet, with which they surround blood vessels. Fibrous astrocytes are found in white matter in close contact with axons and in association with the nodes of Ranvier, projecting longer and thinner processes than those of protoplasmic astrocytes (Butt, 1994).

Along with this classical characterization, other different cellular types are identified, such as retinal Muller radial glial cells, tanycytes in hypothalamus, pituicytes in the neuro-hypophysis, ependymocytes, choroid plexus cells and retinal pigment epithelial cells, which line the ventricles or the subretinal space (Reichenbach, 2005).

Astrocytes in vitro can be stained with their specific marker glial fibrillary acidic protein (GFAP), which also stains astrocytes in pathological conditions characterized by gliosis (Bignami, 1972).

Astrocytes participate in controlling CNS homeostasis at various levels: molecular, through the regulation of ion, neurotransmitter and neuro-hormone concentrations (Newman, 1995; Danbolt, 2001); metabolic, being involved in the accumulation of energy substrates fundamental for neuronal viability (Magistretti, 2006); cellular, with direct involvement in neurogenesis, neural cell migration, synaptogenesis/synaptic pruning and shaping of the microarchitecture of grey matter (Nedergaard, 2003); of organ, in terms of formation and maintenance of the BBB (Abbott,2005).

Astrocytes are metabolically coupled with neurons. They uptake glucose from the capillaries and convert it into lactate, a neuronal metabolic substrate. Lactate is then released by astrocytes and taken up by neuronal cells (Tsacopoulos, 1996).

Astrocytes define anatomical boundaries in the CNS and one of their major functions is supporting neuronal transmission by maintaining local ion and pH homeostasis. They have functional neurotransmitter receptors and can buffer neurotransmitters, such as glutamate or GABA, and modulate transmission (Kang, 1998).

Astrocytes also regulate the function and integrity of the BBB by surrounding the CNS capillaries with end-feet.

Another important characteristic of astrocytes is the release of trophic factors, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) that support the survival of neurons (Dreyfus, 1999).

Recent findings show that astrocytes produce and release various molecular mediators, such as prostaglandin E2 (PGE_2), nitric oxide (NO) and arachidonic acid, which can control CNS blood vessel diameter and thus blood flow (Gordon, 2007). Furthermore, they appear to be primary mediators of the changes in local CNS blood flow in response to changes in neuronal activity. Increase in local neuronal activity induces astrocyte Ca^{2+} signals that, in turn, trigger release of vasoactive substances from the end-foot, thereby regulating the local blood flow (Koehler, 2009).

Astrocytes play a fundamental role in synaptic transmission and plasticity. Their processes wrap and isolate individual synapses, thus providing for spatial specificity and local metabolic support that are critical for synaptic transmission (Bourne, 2008). In addition, polypeptide cytokines such as tumor necrosis factor alpha (TNFα) produced by astrocytes as well as microglia can influence homeostatic synaptic scaling by inducing the insertion of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors at post-synaptic membranes (Stellwagen, 2006).

Finally, astrocytes are critical for maintaining glutamatergic transmission by recycling glutamate through the glutamate–glutamine shuttle for subsequent reconversion into active transmitter in the synaptic terminal (Sattler, 2006).

During pathological conditions of the brain, astrocytes are able to respond promptly to neuronal injury, undergoing changes in their phenotype according to a process known as “astrocyte activation” or “reactive astrogliosis”. They up-regulate the expression of intermediate filaments, such as GFAP, proliferate and form a dense network of hypertrophic cells, filling the gaps that result from neuronal or glial degeneration. Reactive gliosis eventually forms the so called glial scar, a structure that follows brain injury or neurodegeneration and that is important to control damage at the site of insult, by suppressing its expansion to neighboring cells (Reier, 1988).

Astrocyte activation is principally triggered by pro-inflammatory cytokines, which are released by microglia that has been activated by various pathological stimuli. The

cue can be represented either by an acute injury, as in case of acute lesions of the brain parenchyma, such as trauma, ischemia, bacterial and viral infections, or by chronic conditions, as it happens during neurodegenerative diseases, as well as other age-related alterations (Liu, 2011).

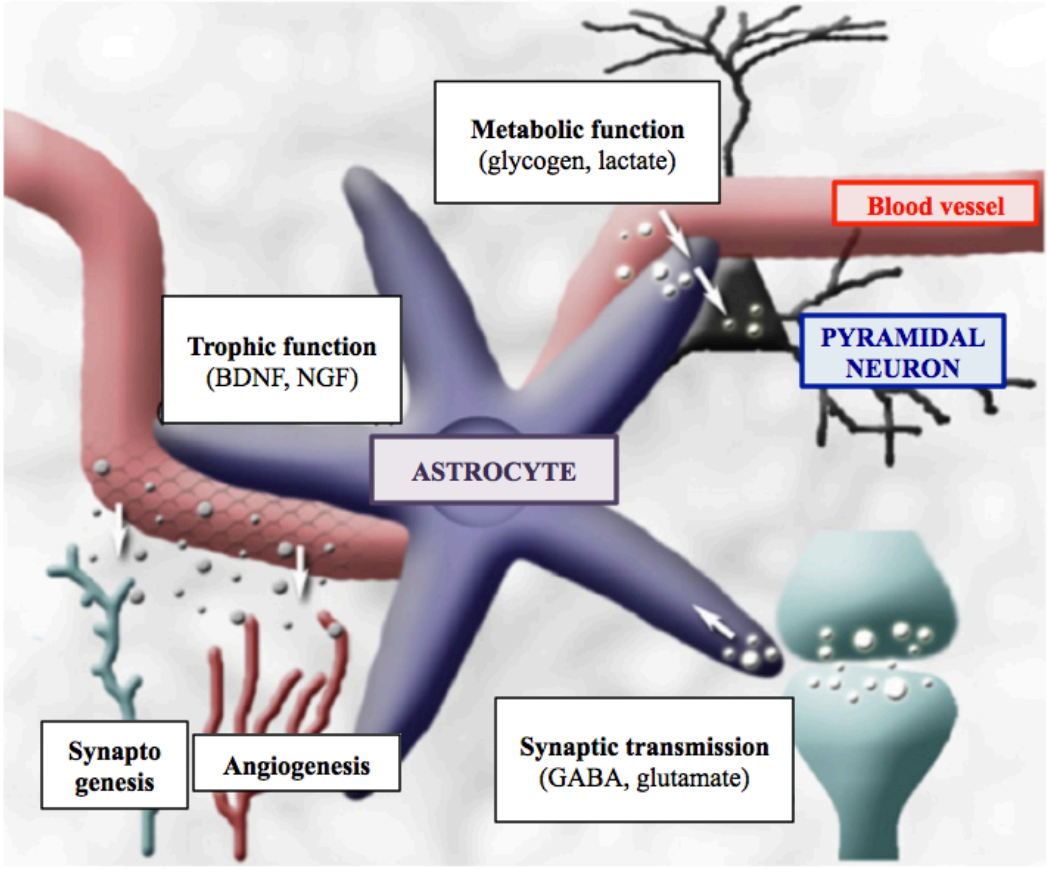


Fig. 1.2: General functions of astrocytes

Astrocytes play several fundamental roles in sustaining neuronal viability. They provide metabolic support to neurons, by supplying them energy substrates (glycogen, lactate) in order to satisfy the high metabolic demand of the brain. They have important functions in synaptic transmission and plasticity through release and uptake of neurotransmitters. They participate to synaptogenesis, angiogenesis and BBB formation and preservation. They release trophic factors (NGF, BDNF), necessary to the maintenance of neuronal survival (modified from Wang, 2008)

1.2.2 Crosstalk between astrocytes and microglial cells

In general, activation refers to an enhanced ability of a cell to perform a function beyond that present in the basal state. The activation of microglia and astrocytes is complex. They proliferate, phagocytose, and release pro-inflammatory cytokines or growth factors (Kettenmann, 2008).

Neurons can activate glia via various neurotransmitters or modulators, such as glutamate, fractalkine, nitric oxide, and other molecules (Liu, 2006). Conversely, the activated glial cells affect neuronal functions and may contribute to the development of various injuries and disorders.

Activation of microglia and astrocytes occurs at various stages in several neurodegenerative diseases. For example, it has been reported that in experimental autoimmune encephalomyelitis (EAE), microglia proliferate at the initial stage while astrocytes start to markedly respond at the late recovery stage (Matsumoto, 1992). Similarly, in Alzheimer's Disease (AD), astrocytes are activated following microglial activation when human-derived dense-core amyloid plaques (typical of this pathology) are injected into rat brain (Frautschy, 1998).

In the activation process, the release of pro-inflammatory mediators is considered closely involved in the activation process and of essential importance in the pathogenesis of various neurodegenerative diseases. Among the molecules that are released during pathological conditions, IL1 has a pivotal role, because of its ability to induce up-regulation of pro-inflammatory mediators, such as IL6 and TNFα (John, 2005). Moreover, IL1 seems to be a crucial target in the interplay between astrocytes and microglia. Microglia is the main source of this cytokine. IL1 positive cells have been reported to overlap with ionized calcium binding adaptor molecule (IBA1, a marker only expressed by microglial cells) in a mouse model of CNS trauma (corticectomy injury), while GFAP positive cells were not present (Herx, 2001), thereby confirming microglia as the only source of this pro-inflammatory mediator. We can therefore envisage a close interdependence between these two types of glial cells, with activated microglia that facilitate astrocytic activation and activated astrocytes that, in turn, regulate microglial activities also favoring further spreading of microglial activation.

Very interestingly, astrocytes play a dual role in CNS inflammatory diseases, either exacerbating (by enhancing immune responses), or limiting (by exerting an inhibitory effect on activated microglia) CNS inflammation. For instance, astrocytes release mediators, such as transforming growth factor β (TGFβ) or PGE_2, that are reported to reduce microglia activation (Ramirez, 2002). Among their effects there is the downregulation of the expression of molecules associated with antigen presentation and the negative feedback on the production of pro-inflammatory cytokines, NO and reactive oxygen species (ROS) (Herrera-Molina, 2005).

An important question is how these two totally opposite actions may coexist. The degree of inflammation seems to be crucial; it was reported that in the presence of strong inflammatory stimuli, astrocytes are unable to inhibit NO production from microglia. Analogously, also the duration of the stimulation appears to be relevant for the outcome of the astrocytic effect on microglial modulation.

The presence of microglia in the experimental system should not be neglected or overlooked in studying in vitro the involvement of astrocytes in the immune response. In many situations, the cellular responses observed in vitro and attributed to astrocytes, might actually due to microglial response. For example, there is ample literature on iNOS expression and subsequent NO production after exposure of astroglial-enriched cultures to bacterial lipopolysaccharide (LPS) activation; nonetheless it was shown that iNOS is predominantly expressed in microglial cells (Saura, 2007).

Until now few studies have explored the inhibitory effect of activated astrocytes on microglial activities. In particular, it is not well clear whether activated astrocytes can have a feedback role on microglia, resulting in inhibition of CNS inflammation, or whether they can amplify the inflammatory outcome, thus exacerbating neurotoxicity.

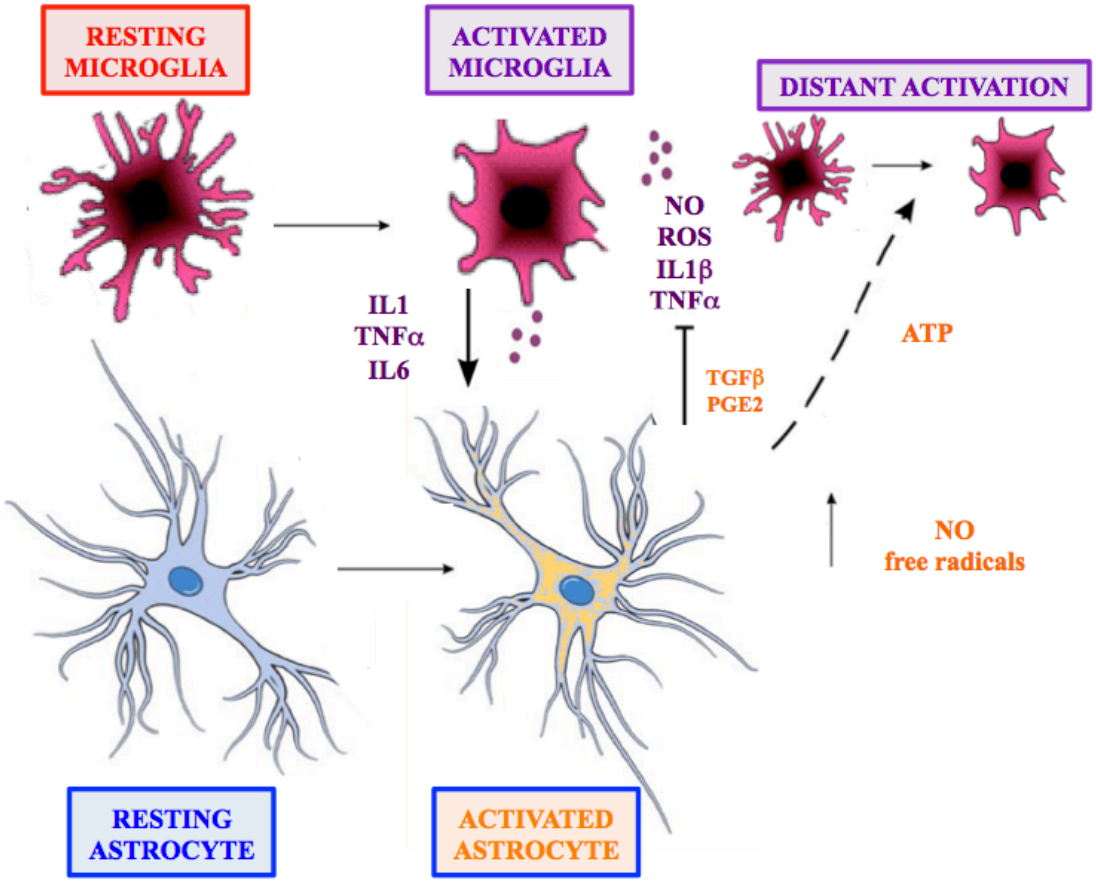


Fig. 1.3: Interplay between astrocytes and microglial cells

Astrocyte activation is mainly triggered by pro-inflammatory cytokines released by microglia stimulated by different pathological stimuli. Activated astrocytes can modulate microglia activation in a negative way, through release of molecules like TGFβ and PGE_2, or they could promote the neurotoxic context through the production of NO and free radicals. Moreover, astrocytic calcium waves could increase the release of ATP, thus triggering distant microglial activation. (Adapted from Liu, 2011)

1.3 Microglia: biology and functions

Microglial cells are recognized as the resident macrophage population of the CNS, and are distinguished from other glial cells by their origin, morphology, gene expression pattern and functions (Kettenmann, 2011). Microglia constitute 5–20% of total glial cells in rodents (about 12% in human), depending on the specific region of the CNS (Lawson, 1990). In contrast to neurons and other glial cells, microglia are of hematopoietic origin and act in response to pathogen infection and injury. Differently from other populations of macrophages, they have a characteristic ramified morphology and communicate with surrounding cells.

Microglia respond to a large spectrum of stimuli by adopting an amoeboid activated phenotype and producing many pro-inflammatory mediators, such as cytokines, chemokines, reactive oxygen species and nitric oxide, thus contributing to the clearance of pathogen infections or neuronal debris. However, prolonged or excessive microglia activation may result in pathological forms of inflammation that promote the progression of neurodegenerative and neoplastic diseases (Glass, 2010; Perry, 2010).

1.3.1 Origin and historical view of microglia

Microglia cells were first recognized by Nissl, who named them “staebchenzellen” (rod cells) because of their rod-shaped nuclei and also “reactive neuroglia” to distinguish them from the other unresponsive glial cells (Nissl, 1899). However, it was in 1927 that Del Rio Hortega described them as a defined cellular element of the CNS, different from astrocytes and oligodendrocytes after a series of studies using his silver carbonate impregnation staining method. He named the new cellular class as “microglia”, and the individual cells “microgliocytes”. He observed that microglia possess elongated bipolar cell bodies with spine-like processes that branch out perpendicularly. He supposed that microglia might originate from mononuclear cells of the circulating blood and have the ability to change from the resting ramified form into amoeboid macrophages (Del Rio Hortega, 1932). More in detail, he postulated statements that still perfectly hold:

- microglia enter all brain regions during development using vessels and white matter

tracts as guiding structures for migration;

- these invading cells have an amoeboid morphology and are of mesodermal origins, but they transform into a ramified branched morphological phenotype in mature brain (known today as resting microglia);
- they are found dispersed throughout the mature CNS, but each cell seems to patrol a defined territory;
- after a pathological event these cells acquire an amoeboid morphology which is similar to the one observed early in development;
- microglial cells have migration, proliferation and phagocytosis capability.

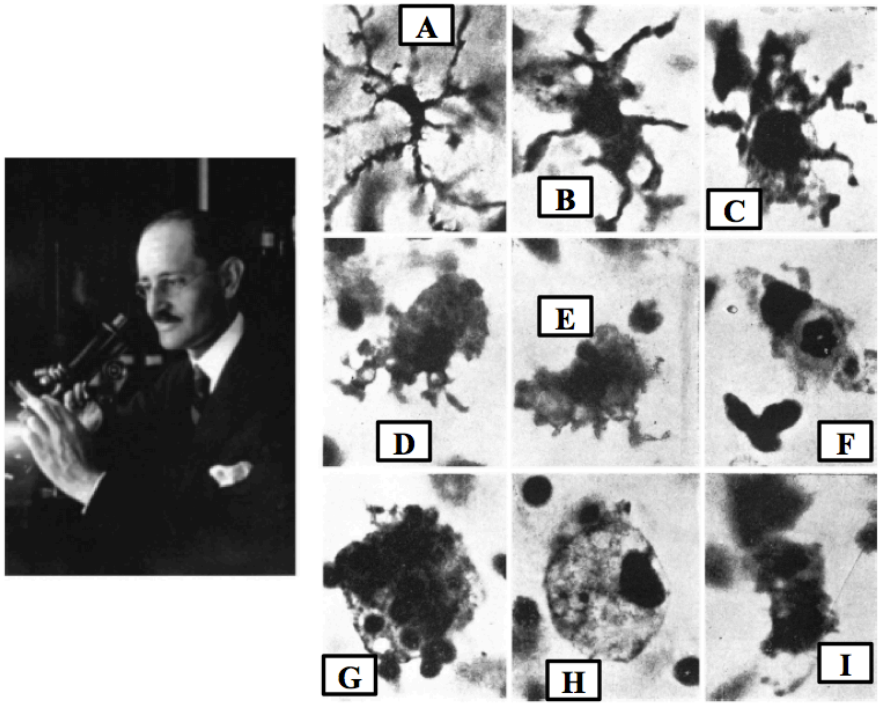


Fig. 1.4: Microglial cells discovered by Pio del Rio-Hortega

Picture of Pio Del Rio-Hortega (1882–1945) and images of microglia evolution during its phagocytic activity: A) cell with thick, rough prolongations; B) cells with short prolongations and enlarged cell body; C) hypertrophic cell with pseudopodia; D) and E) amoeboid and pseudopodic forms; F) cell with a phagocytosed leukocyte; G) cell with numerous phagocytosed erythrocytes; H, fat-granule cell; I, cell in mitotic division (modified from Del Rio Hortega, 1932).

The origin of microglia has been at the center of a debate for several decades (Cuadros, 1998; Kaur, 2001). These cells have been proposed to derive, alternatively, from neuroectoderm (like astrocytes or oligodendrocytes), vascular adventitia, an intrinsic population of hematopoietic stem cells resident within the CNS, and from circulating blood monocytes. Today there is a general consensus that microglia are of mesoderma/mesenchymal origin: they are generated from peripheral circulating blood monocytic progenitors that cross over the wall of blood vessels and populate the nervous system (Perry, 1985). However, unlike other resident monocyte populations, microglia appear to constitute a self-renewing population throughout adulthood, separated from the bone marrow-derived monocyte lineage. Until recently, the large increase in the local microglial population (microgliosis) following CNS insult was believed to at least partly originate from bone marrow-derived progenitors (Djukic, 2006).

Resident microglia in the adult have a slow turnover at rest and are capable of proliferation and self-renewal. Under pathological conditions, there is a varying degree of recruitment of peripheral bone marrow progenitors to the adult nervous system (Beck, 2003; Priller, 2001). This means that it is not expected a significant turnover from the periphery in the healthy CNS (Lassmann, 1993). Indeed, in adult animals there seems to be very little exchange between brain parenchyma and blood. However, the lack of differential expression of cell surface markers, between mononuclear phagocytes that infiltrates the adult nervous system under pathological conditions and resident microglial cells makes difficult to discriminate their tissue source and cell lineage (Rezaie, 2002).

The identification of microglial cells is nowadays a very debated issue. Moreover, the suitability of the various markers is determined by the ability i) to discriminate microglia from other CNS-resident cells, like neurons, astrocytes, oligodendrocytes, or endothelial cells; ii) to distinguish parenchymal microglia from other resident or infiltrating monocytes/macrophages; and iii) to either reveal the entire population of microglia or to display a specificity for certain activity states.

1.3.2 Morphology of microglial cells

During early postnatal development, monocytes infiltrate the brain and transform into amoeboid microglia, morphologically characterized by a large cell body and short processes (Ling, 1993). This allows them to move within the brain, by pruning axons where appropriate and phagocytosing apoptotic cells and other debris associated with CNS development (Mallat, 2005). Within weeks of their appearance in the CNS, microglia adopt a more ramified, resting phenotype. Their morphology in the healthy tissue of the nervous system does not suggest an immediate association with a macrophagic nature. The presence of cellular processes branching off from the soma is typical for "ramified" microglia. This term has been almost evenly used with "resting" microglia, pointing out to the intimate link between their morphology and their role of sensors of neuronal structural and functional integrity (Streit, 1992).

In vivo two-photon imaging of microglia at rest in the mouse neocortex has revealed, surprisingly, high dynamics of their processes (Nimmerjahn, 2005). Resting microglia continuously supervise the microenvironment interacting with other cortical elements, rather than passively receiving potential danger signals. Upon different stimuli, the process of microglial transformation from the resting to the activated states is accompanied by marked morphological changes (Lynch, 2009). Microglia reduce the complexity of their shape by retracting the branches of their processes. Several steps and intermediate stages can be identified, including the process of withdrawal, transition or hyper-ramification, and subsequent formation of new protrusions as well as motility and movement in the tissue. Interestingly, also in aging, microglia gain morphological characteristic of senescence and functional deterioration (Miller, 2007).

In vitro, microglial cells usually do not usually have the ramified structure typically seen in the healthy CNS. They show heterogeneous shapes, ranging from spindle and rod-shaped or amoeboid versions with short thick processes expanding as lamellipodia from round cells. A morphological reorganization can still be imposed by treating with typical activating agents, such as bacterial lipopolysaccharide (LPS), a constituent of the outer cell wall of gram-negative bacteria (Abd-el-Basset, 1995). According to the stimulus, a different activated phenotype could be triggered (Nelson, 2002); moreover, it is known that the various activated forms may interchange, while progression from

the ramified microglia to the amoeboid macrophage-like appearance is thought to occur as a stereotypic sequence (Stence, 2001).

Although the fast and drastic transformations in cell shape suggest that they are required for functional adjustments (for example, filopodia protrusion and dynamic rebuilding are needed for motility and directed migration, as well as appearance of "foamy" cells points to phagocytic activity), morphology does not necessarily provide a reliable indication of functional orientation. On the contrary, certain gene inductions or activities can occur even in the absence of obvious morphological transitions, whereas a ramified shape can be experimentally induced under conditions that do not support ramification (Suzumura, 1991). As a consequence, the loss of ramified toward a more amoeboid cell conformation does not tell much about the actual reactive phenotype.

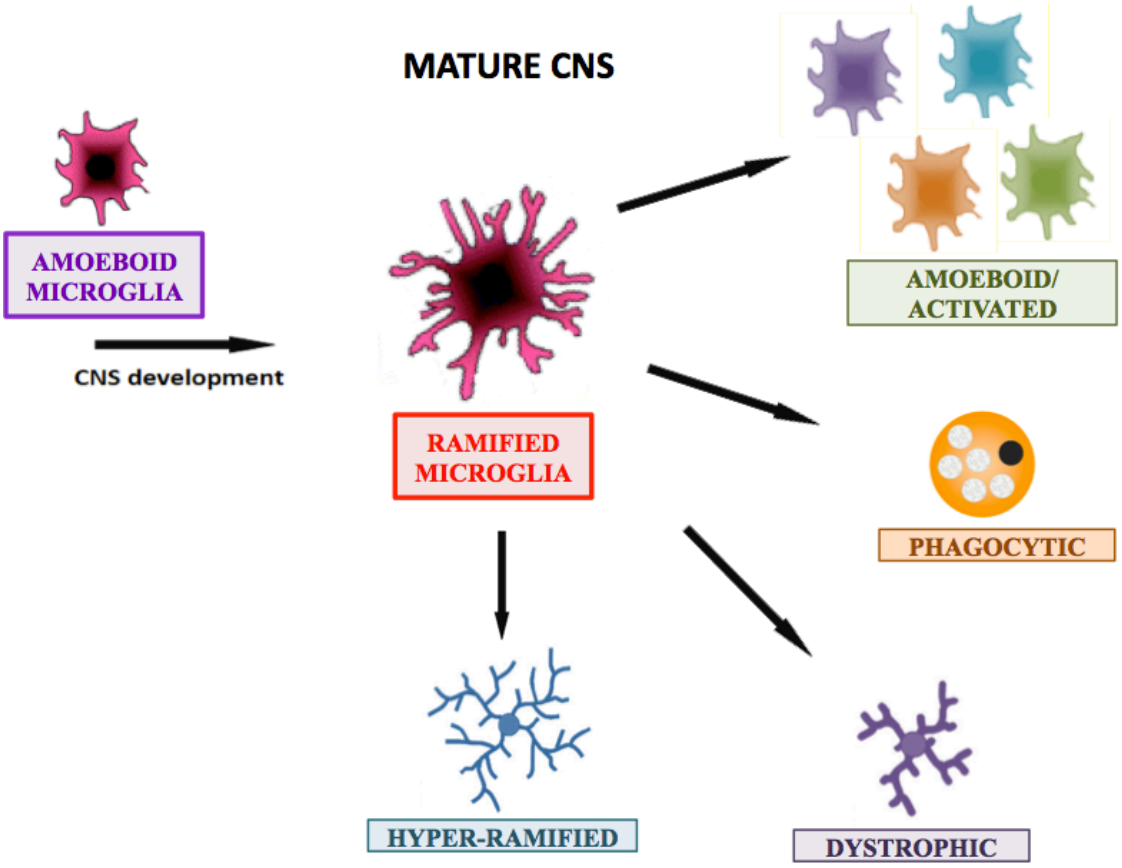


Fig. 1.5: Diagrammatic representation of microglial morphology

During CNS development, monocytes infiltrate the brain and transform into amoeboid microglia. In the mature adult CNS, microglia exist in a ramified state and assume a rounded phenotype only when activated. The nature of different stimuli that trigger microglia activation promotes the heterogeneity of phenotypes. Microglia become phagocytic under conditions of neuronal death and degeneration. In senescence microglial cells acquire a dystrophic morphology indicating functional deterioration.
(adapted from Kreutzberg, 1996 and Perry, 2010).

1.3.3 Functions of microglial cells

As previously mentioned, during development, microglia (with an amoeboid phenotype derived from the myelomonocytic lineage) are produced in the bone marrow, invade the CNS tissue and actively migrate within the brain parenchyma to colonize all regions of the brain (Davoust, 2008). Amoeboid microglia are also believed to play a role in promoting axonal migration and growth (Rakic, 2000). After colonizing the CNS, amoeboid cells acquire a ramified phenotype and extend long processes.

Microglia act as sensors of brain pathology, ready to react in response to injury or invasion of a 'foreign' body into the CNS. In contrast to the microglial cell body, which remains stable, their processes are highly motile and dynamic in vivo, constantly extending and retracting (Davalos, 2005). This dynamic movement can be viewed as a constant surveillance of the microglial cell territory, suggesting that the term 'surveying' microglia successfully describes (better than "resting") their function in the healthy brain.

Although their physiological functions are only partially understood, a microglial role is well recognized in the pathological mechanisms of several neurological disorders, including acute and chronic neurodegenerative disorders of the immature and mature CNS, is well recognized.

Depending on the type of injury, as well as on the activating or inhibiting microenvironmental conditions, microglia can exacerbate a pathological context and the subsequent neurodegeneration, but can also play an important role in the protective mechanisms underlying tissue repair and regeneration.

Microglia acquire an activate phenotype when neurons are damaged, as a result of aging, insults or neurodegeneration, and release of specific molecules, such as ATP, neurotransmitters, growth factors or cytokines. Microglia are also alerted when they encounter molecules that are not found in the healthy CNS, such as blood clotting factors, intracellular constituents released by necrotic cells (i.e., RNA, DNA), externalized phosphatidylserine on apoptotic cells, immunoglobulin-antigen complexes, opsonizing complement, abnormally folded proteins (i.e., protein aggregates), or pathogen-related structures (Hanisch, 2007).

Based on the activation stimuli, microglia are induced to perform phagocytic activity

in order to clear apoptotic cells or abnormal proteins, or to act as secreting cells. Depending on the environment in which they are activated, microglia can either take on a “classical activated” phenotype (analogous to the macrophagic M1), characterized by the release of toxic pro-inflammatory molecules, or an “alternative activated” (macrophagic M2) one (Mantovani, 2004). M2 microglia are typically considered less inflammatory than M1 cells and are characterized by reduced nitric oxide secretion and increased anti-inflammatory cytokine production. Accordingly, microglial heterogeneity exists during neurodegenerative disease and may influence disease outcome (Colton, 2006).

Microglia have the remarkable ability to recognize a wide range of signals that indicate a threat to the structural and functional integrity of the CNS through various receptors. For instance, injured neurons release a variety of molecules able to trigger microglial responses; among the others, ATP and chemokine CXC motif ligand 10 (CXCL10), attract microglia via activation of purinoreceptors (Davalos, 2005) and chemokine CXC motif receptor 3 (CXCR3; Rappert, 2004), respectively.

In this context, astrocytes are also involved in the release of different factors, able to modulate microglial responses.

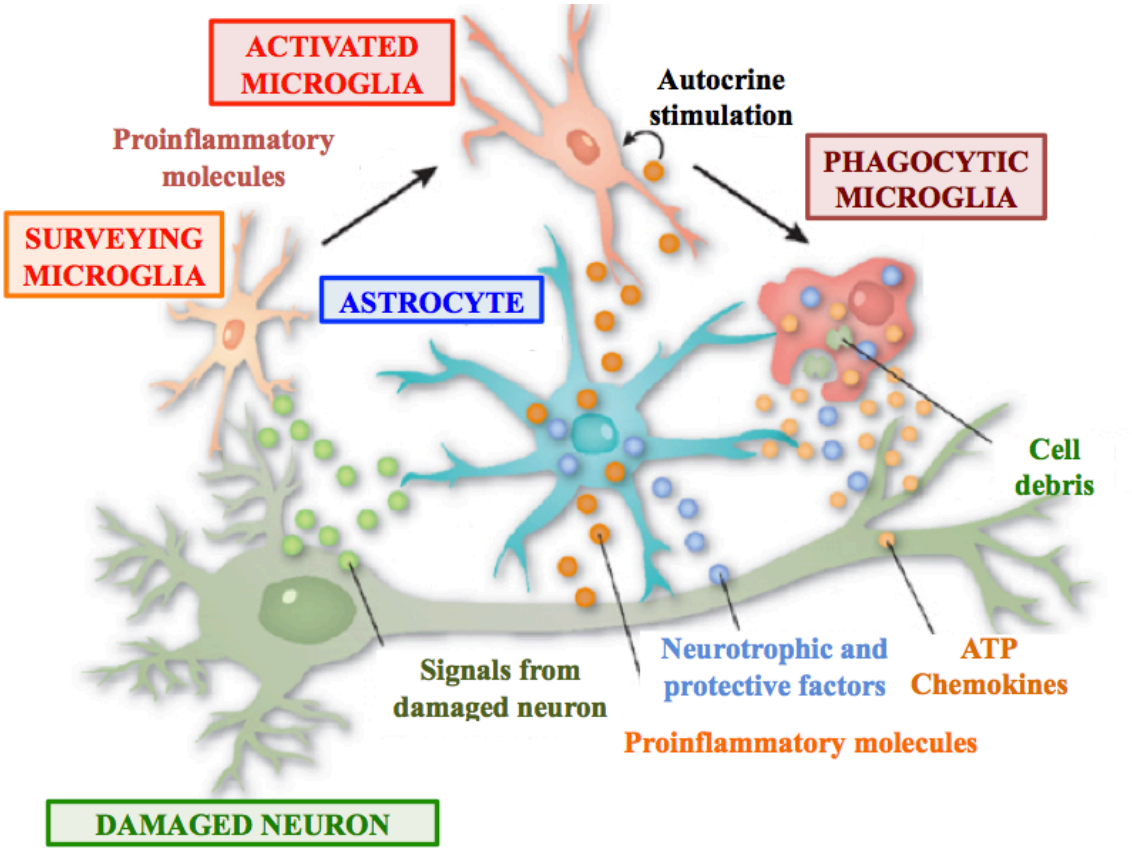


Fig. 1.6: Microglial general functions

Microglial cells continuously supervise the microenvironment and dynamically interact with other nervous and glial cells. Molecules released by damaged neurons trigger a microglial response, which can mediate the regeneration process through phagocytosis, or the amplification of the neurotoxic context. (Modified from Monk 2006)

As main cells of innate immunity of the CNS, microglia constitutively express the most important immune receptors (MHC I and II, chemokine receptors) at low levels. During the process of activation, all the immunologically relevant molecules are upregulated, and the appropriate antigen is presented via MHC II. Additionally, it has recently been shown that microglia are able to cross-present exogenous antigens on MHC I to CD8+ T cells (Beauvillain, 2008).

Pattern recognition receptors (PRRs) are generally constitutively expressed by microglial cells and are crucial to the innate immune response (Akira, 2006). They include specific Toll-Like receptors (TLRs), nucleotide-binding oligomerization domain proteins (NODs) and scavenger receptors. In response to injury, microglia express most of the TLRs, recognize pathogens and initiate the pro-inflammatory response. All toll like receptors (TLR1-TLR9) can be expressed in human microglia (Bsibsi, 2002). TLR4, which is the receptor for LPS, was shown to be exclusively expressed by microglia in the CNS (Lehnardt, 2002).

Stimulation of microglia with TLR agonists, including LPS for TLR4, peptidoglycan for TLR2, poly (I:C) for TLR3, and CpG DNA for TLR9, leads to increased secretion of most cytokines expressed by microglia with consequent neurotoxicity in neurodegenerative disease and modulation of the immune response in neuroinflammation (Lehnardt, 2009).

Stimulation of pattern recognition receptors leads to the activation of signal transduction pathways that regulate diverse transcriptional and post-transcriptional processes. For example, the TLRs couple to signaling adaptor systems that are defined by the MyD88 and TRIF signal adaptor proteins resulting in activation of downstream kinases including IkB kinases and MAP kinases. These, in turn, control the activities of multiple, signal dependent transcription factors that include members of the NF-kB, AP-1, and interferon regulator factor (IRF) families. These factors work in a combinatorial manner to regulate hundreds of genes, depending on the target cell.

Microglia can also sense neuronal activity through neurotransmitter receptors, such as α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic glutamate receptors, γ-aminobutyric acid B (GABA-B), purinergic, adenosinergic, cholinergic, cannabinoid, adrenergic and dopaminergic receptors, as well as receptors for neuroactive peptides. Each neurotansmitter receptor is uniquely involved in

microglial function (Pocock, 2007).

Along this line, data from the literature demonstrate that glutamate receptors present on microglia act as a link between inflammation and excitotoxic brain damage (Tahraoui, 2011). Of note, in vitro, microglia express the glutamate transporter (GLT-1) and have been shown to both take up and release glutamate (Persson, 2005). Glutamate is the main excitatory neurotransmitter in the CNS and its levels in the extracellular space are tightly regulated, mostly by astrocytic activity. The fact that microglia have the potential to regulate glutamate levels suggests that they participate in regulating synaptic activities and plasticity.

The microglial cytokines IL1β and TNFα have been shown to modulate the expression, activity, and trafficking of neuronal glutamate receptors, which are determinants of synaptic strength (Beattie, 2002). However, in culture conditions, microglia do not release detectable levels of these cytokines without the presence of activating agents. Thus it remains unclear whether microglia in vivo are bona-fide modulators of synaptic activity.

The main question that arises is whether activated microglia contribute to neurotoxicity in brain injury, inflammation and neurodegenerative diseases, or whether their presence and activation have neuroprotective effects. In neurodegenerative diseases, persistent, chronic microglial activation, accompanied by sustained secretion of inflammatory mediators may exacerbate neurotoxicity and disease processes. In neuroinflammation, microglia seem to play a dual role, being involved in the initial inflammatory response where they act as antigen presenting and debris phagocytosing cells, but also exhibiting a persistent activation phenotype in disease progression.

Given the progressive and cumulative contribution of microglial activation throughout the course of different neurodegenerative disorders, monitoring microglial activation could be an indicator of disease progress and severity or could be an early diagnostic marker. At the same time, clarifying which microglial processes contribute to neurotoxicity and which have a protective effect could lead to potential therapeutic manipulation of these activation pathways, aimed at limiting the former and enhancing of the latter.

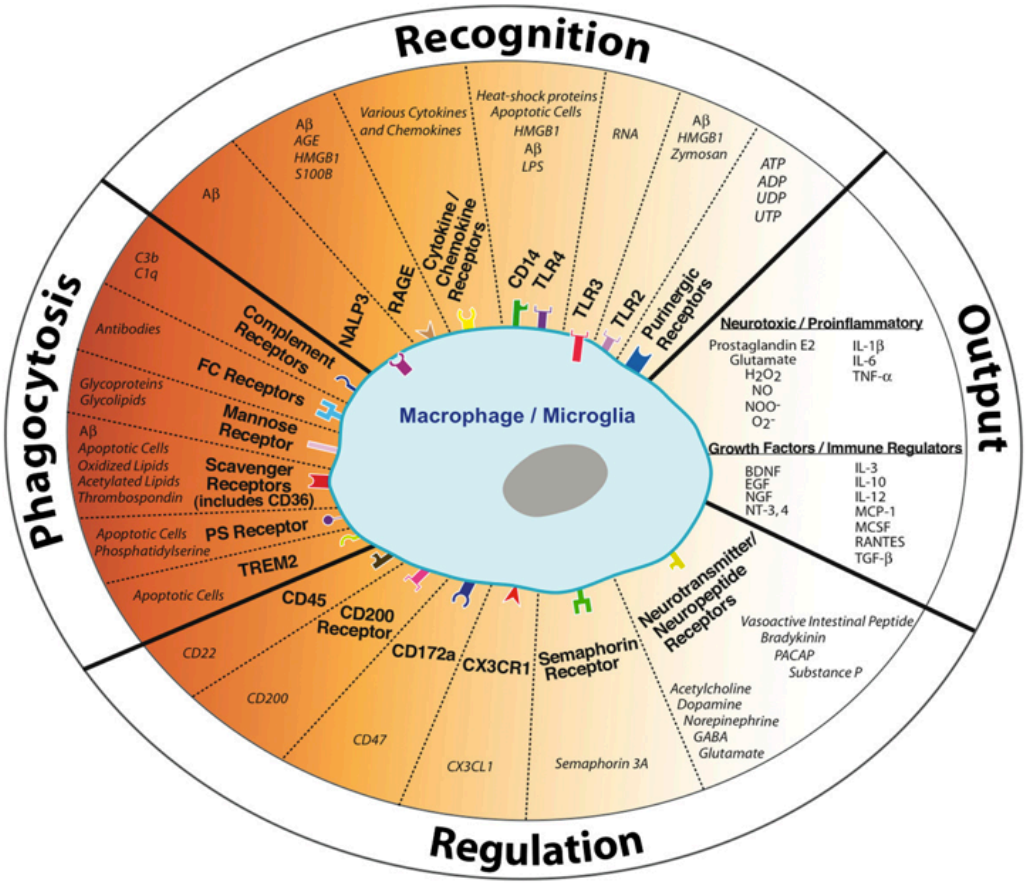


Fig. 1.7: Regulation of microglial functions in CNS

Microglia are activated following the stimulation of various recognition or phagocytic receptors. This activation state is subsequently controlled by neurons, which secrete or express numerous regulatory ligands. The end result of these interactions is the release of cytokines, neurotoxic substrates, or growth factors by microglia or the activation of cellular pathways including phagocytosis. Aberrant function of these pathways can result in significant degeneration during aging or disease.

(Modified from Lucin, 2009)

1.4 Microglia activation

Upon binding to their respective receptors on microglia, molecules that signal danger can activate the intracellular pathways that result in the activation and translocation of several transcription factors. Signal transducers and activators of transcription (STAT), cAMP response element binding protein (CREB), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) are among the microglial transcription activated by danger signals (Potucek, 2006). Thereafter, the genes that are transcribed can define the function of activated microglia.

Microglia can be rapidly activated by various environmental changes. As already pointed out, the process of microglial activation is associated with proliferation and transformation into 'reactive' microglia with distinct response phenotypes (Garden, 2006). Activated microglia proliferate, migrate to the site of damage and secrete pro- and/or anti-inflammatory cytokines and chemokines, oxidative stress-inducing factors such as NO as well as growth factors. Whether microglia activation has a beneficial or harmful function depends on several factors: the kind of stress and damage signals, the duration of an insult, the microenvironment, the interaction with other cell types and, interestingly, even the age of an organism (Walter, 2009). In the case of acute neuronal death, microglia can function as brain macrophages and phagocyte cell debris (Graeber, 2010).

Excessive acute, sustained (chronic) or maladaptive responses of microglia may lead to substantial impairment of neurons and glia. Failure of protection and an active contribution to damaging cascades have been attributed to activated microglia in many pathologic scenarios. However, such data probably underestimate the microglial capacity to safeguard and stabilize the CNS.

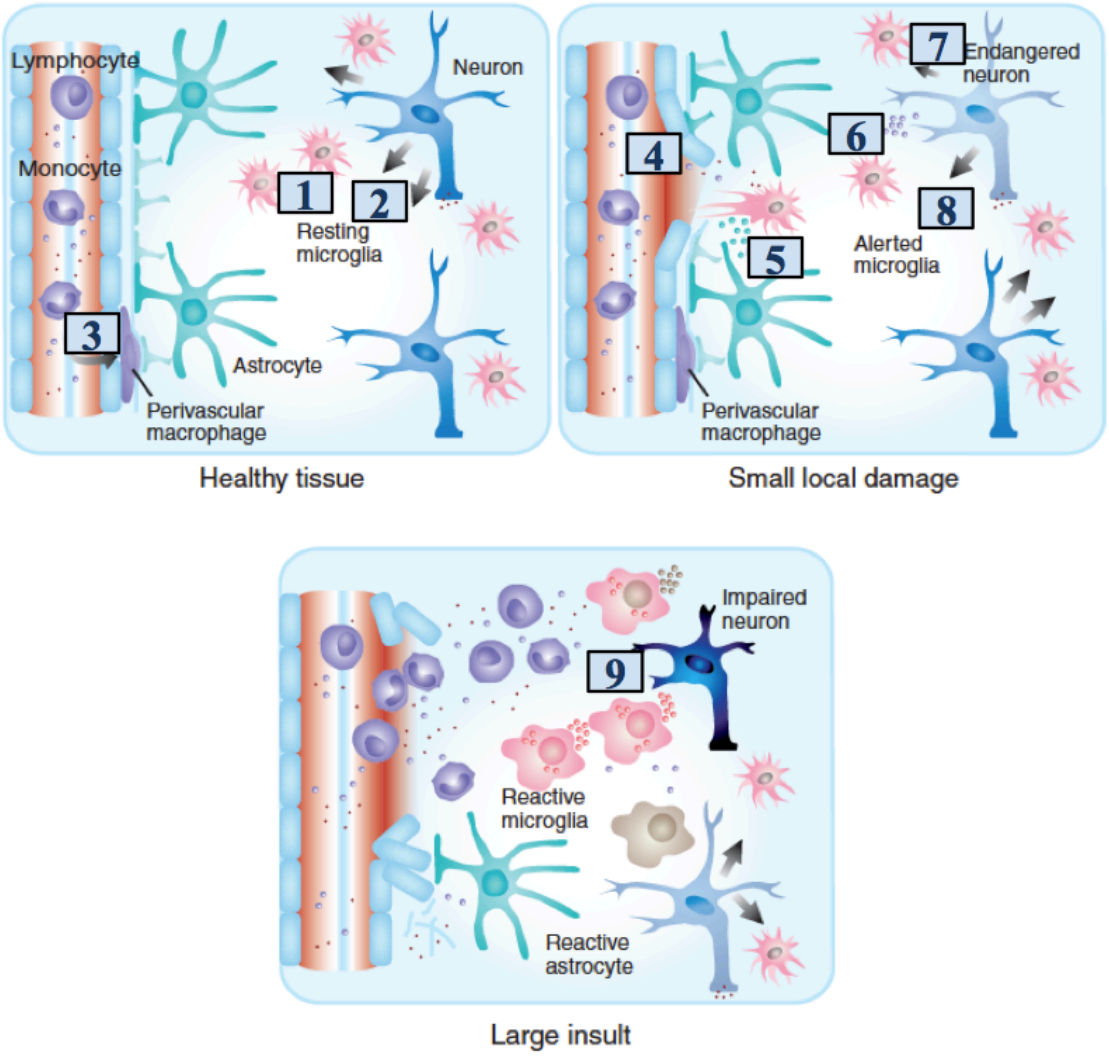


Fig. 1.8: Multiple activation states of microglia cells.

1. Microglial processes undergo continuous rebuilding to allow efficient scanning of CNS territory. Equipped with receptors for a plethora of molecules, they can immediately sense signs of disturbed structural and functional integrity. **2.** Neurons may also deliver signals, which keep microglia in this surveillance mode, indicating normal function. **3.** Perivascular macrophages remain in close association with blood vessels. **4.** Upon detection of tiny vascular or tissue damage, microglia can rapidly respond with a directed reorganization of processes and a change in phenotype. **5.** The response is supported by neighboring astrocytes that release pro-inflammatory mediators. **6.** Microglia can produce neurotrophic factors to support endangered neurons. **7-8.** Disruption of ongoing communication would allow an endangered neuron to enroll microglia. Such neurons can also secrete signals indicating disturbed functions by using molecules that are not usually released. Microglial cells limit further damage and restore normal homeostasis. **9.** Stronger insults to the CNS trigger more drastic changes in the functional phenotype of microglia. Depending on the nature of the stimuli and their context, microglial cells acquire and adapt their reactive behavior. (Modified from Hanisch and Kettenman, 2007)

As mentioned before, microglia/macrophages can be classified into at least two phenotypical subsets with distinct molecular phenotypes and effector functions, depending on the activation pathway. The 'classically activated' pro-inflammatory M1 microglia/macrophages, induced by LPS or by the pro-inflammatory cytokine interferon γ (INFγ) produce high amounts of oxidative metabolites (NO and superoxide), proteases and pro-inflammatory cytokines. They play a central role in the host defense against pathogens and tumor cells, and they can also damage healthy cells such as neurons and other glial cells. For instance, exposure of monocytes, macrophages and microglia to LPS activates TLR4 – with the participation of other accessory proteins such as LPS-binding protein (LBP) and CD14 - and elicits multiple downstream signaling pathways that include the activation of the serine/threonine mitogen-activated protein kinase (MAPK) family, and the subsequent phosphorylation of their target transcription factors. In addition to the MAPKs, also NF-κB and protein kinase C (PKC) signal transduction pathways are activated by LPS (Guha and Mackman, 2001). A large and diverse group of nuclear transcription factors, such as AP-1, CREB response element (CRE), serum response element (SRE) and NF-κB, are the downstream targets of these signaling pathways and act to coordinate the induction of multiple genes encoding inflammatory mediators and co-stimulatory molecules (Yang et al., 2003).

At difference with the M1 phenotype, the 'alternatively activated' M2 microglia/macrophages have an anti-inflammatory function, thus down-regulating inflammation and promoting tissue remodeling/repair and angiogenesis. They are commonly induced by IL4 and IL13. It is feasible that, in order to stop the "killing" phase of classically activated microglia/macrophages and to restore the tissue homeostasis after injury, a change of the activation state into a reparative one (M2) is necessary. It is not clear whether deactivated microglia return to the same functional state as resting microglia or retain some sort of memory of their first activation.

The M1/M2 classification is, of course, a simplification and a number of other intermediate phenotypes have been described (Colton, 2009). Although they have not been yet completely characterized, activation markers, specific for certain microglial "states" and with an identified spectrum of released factors, have been recognized.

In 2003, Gordon and colleagues termed "acquired deactivation" a second microglia activation state associated with an anti-inflammatory phenotype. Alternative activation

and acquired deactivation both down-regulate the innate immune responses and have similar, but not identical, gene expression profiles. The most prominent difference is that acquired deactivation is induced by exposure of macrophages to apoptotic cells or to TGFβ and/or IL10 (Gordon, 2003). Overall, when looking at the relationship between activation phenotypes and pathological stimuli, it appears that the regulation of microglial cell phenotype can be regarded as a potential approach for therapeutic intervention.

Activation state	Specific induction agent	Primary signaling mediator	Identifying antigens	Shared antigens	General functions
Classical activation	IFN-γ	STAT1 (STAT4)	IL-12p40, MARCO TNF-α, IL-6, IL-1b	MHCII, C-type lectins, CAT2	Tissue defense, proinflammatory cytokine production, NO production
Alternative activation	IL-4, IL-13	STAT6	MR, AG1, ym1/Chi3L1/L2, lack of NOS2, DC-SIGN, FIZZ1	MHCII, CD163, CD36, C-type lectins, CAT2, IL-1Ra	Tissue repair, anti-inflammatory cytokine production, fibrosis, ECM reconstruction
Acquired deactivation	TGF-β, IL-10, apoptotic cells	STAT3 SMAD	sphk-1/2 IL-4Rα, high IL-10, SOCS3, CCL18	CD163, CD36, C-type lectins, low MHCII, IL-1Ra	Immunosuppression, oxLDL and apoptotic cell uptake

Fig.1.9: Identifying features of the microglial activation states

Expression markers and relative functions of the different putative phenotypes characteristic of the activation process of microglial cells. Based on the inflammatory environment, activated microglial phenotypes can acquire different characteristics triggered by the expression of specific genes and the release of mediators that determine the heterogeneity of the responses (from Colton, 2009).

1.4.1 Microglia activation: neurotoxicity and neuroprotection

Microglia-mediated neurotoxicity contributes to the chronic nature of several neurodegenerative diseases (Gao, 2003). This fact has been most effectively demonstrated in models using LPS. Even though LPS models cannot precisely mimic the conditions under which these glial cells are activated in neurodegenerative diseases, these studies demonstrate that LPS is neurotoxic only in the presence of microglia, indicating a pivotal role of microglia in initiating neuronal damage (Gao, 2002). Indeed, LPS is reported to induce microglia activation in vivo and in vitro and to promote the progressive and cumulative loss of dopaminergic neurons over time (Ling, 2006). Moreover, embryonic exposure to LPS has an impact on microglial activation and neuron survival into adulthood (Carvey, 2006).

Interestingly, some neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is a precursor to MPP+ and causes a permanent Parkinsonian syndrome by destroying dopaminergic neurons in the substantia nigra of the brain, require the presence of neurons to induce microglial activation, and effect their damage through direct action on neurons and indirect over-activation of microglia (McGeer, 2003). In fact, although MPP+ cannot directly activate microglia, addition of these cells to neuronal cultures enhances MPP+-induced toxicity. Furthermore, in several animal studies, MPTP toxicity is significantly reduced in mutant mice deficient in pro-inflammatory factors such as superoxide (Zhang, 2004), prostaglandins (Feng, 2002), nitric oxide (Wu, 2002) and TNFα (Sriram, 2006). These findings, taken together, suggest that microglia amplify neuronal damage induced by pathological stimuli and toxins (Block, 2002).

Endogenous protective regulatory signals in brain have been identified to inhibit microglial activation, such as neuropeptides, cannabinoids, anti-inflammatory cytokines (such as IL10 and TGFβ), estrogens, glucocorticoids. However, it has been proposed that, when the ability to activate these protective mechanisms fails, or when they are engulfed by an excessive inflammatory response, microglia initiate neuronal death and drive the progressive nature of neurodegenerative disease (Polazzi, 2002).

In parallel with their negative or neurotoxic effects, microglia also play a crucial role in the maintenance of neuronal homeostasis (Streit, 2002). Microglia have an important

protective function in brain injury by removing damaged cells, promoting neurogenesis and re-establishing a functional neuronal environment by restoration of the myelin sheath, and the release of neurotrophic factors and anti-inflammatory molecules. These molecules, which are called effectors, include a long list of cytokines, neurotrophins, neurotransmitters, and autacoids. Although some of these molecules have well-defined roles, the majority can be either toxic or trophic to the surrounding target cells depending on the context. For example, under arginine-depriving conditions, microglial inducible nitric oxide synthase (iNOS) produces superoxide anions, which are free radicals that harm many cellular components. The same enzyme also generates nitric oxide (NO) that can cause cell death when converted to the reactive oxidant peroxynitrite. However, NO is also neuroprotective, given its powerful vasodilating action, which is a key factor to rescue the brain tissue in the event of obstructed blood flow (Gibson, 2005). Another factor among the classical pro-inflammatory cytokines of neurodegeneration, TNFα, can act both as a neurotoxic and as a neuroprotective agent. Although TNFα is neurotoxic at high levels, it has been demonstrated to have a neuroprotective effect at low levels, as it has been demonstrated in a study using TNF receptor (TNFR)-deficient mice (Bruce, 1996). This dual role seems to be dependent on the kind of the activated receptor: TNFR1 can induce neurodegeneration, whereas TNFR2 would promote neuroprotection (Fontain, 2002). For this reason, classifying released effectors of microglia based on cytotoxicity alone is erroneous and oversimplifying.

Pro-inflammatory factors, when released systemically, promote an inflammatory process that includes the recruitment of immune cells to the site of inflammation, focal edema, fever, and production of pathogen-killing compounds. The anti-inflammatory effectors, on the other hand, inhibit this inflammatory process. Since the brain contains a very low number of non-microglial immune cells, when the BBB is intact, systemic inflammation and neuroinflammation are coordinated differently. Moreover, the cytotoxic nature of pro-inflammatory cytokines in the systemic context is not always transferable to the CNS setting, as illustrated in the above examples of TNFα and NO.

1.5 Glia activation, neuroinflammation and neurodegenerative diseases

Inflammation is an underlying component of a wide range of neurodegenerative diseases and their associated neuropathology. As mentioned, increasing evidence suggests that microglia and their activation are a key factor in this process. Microglia with a morphologically activated phenotype are present in large number in CNS tissue from patients with chronic neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), prion diseases and multiple sclerosis (MS). A positive feedback loop has been hypothesized: the slow degeneration of neurons and their processes might activate microglia, which secrete neurotoxic molecules. These factors, in turn, promote neurodegeneration, thereby initiating a self-perpetuating degenerative process (Akiyama, 2000).

A very important contribution arises from astrocytes that, once activated by factors released by microglia, might either exacerbate or ameliorate the inflammatory context. Indeed, although some inflammatory stimuli also induce beneficial effects (phagocytosis of debris and apoptotic cells), and inflammation itself is often linked to tissue repair processes, uncontrolled inflammation may result in production of neurotoxic factors that amplify disease states. Numerous negative feedback mechanisms have been identified that serve to attenuate responses to inducers or amplifiers of inflammation. These include induction of proteins that inhibit signal transduction pathways (e.g., SOCS proteins), induction of transcriptional repressors and trans-repressors (ATF3, Nurr1), as well as production of soluble or cell-surface mediators with anti-inflammatory activities. How these negative feedback pathways are integrated with pro-inflammatory signaling pathways, to bring about appropriate resolution of inflammation in any context, remains poorly understood.

1.5.1 Alzheimer's disease

Alzheimer's disease (AD) was first described almost 100 years ago by Alois Alzheimer as a progressive neurodegenerative disorder that gradually destroys hippocampus and cortical regions, leading to a dramatic loss of memory and cognitive abilities. The two major hallmarks of this pathology are represented by the extracellular deposition of the amyloid β (Aβ) peptide in senile plaques and by the intracellular accumulation of neurofibrillary tangles (NFTs) (Carter, 2001).

The causes of AD are still rather poorly understood. While the etiology of the genetic forms has been established, various etiologies have been hypothesized for amyloid plaques, tangle formation, and extensive neuronal death in sporadic AD (e.g. genetic risk factors, Aβ overproduction, Aβ impaired clearance, oxidative stress, vascular issues, etc.). Several lines of evidence point to Aβ as the major driving force in the pathogenesis of AD. The amyloid cascade hypothesis proposes that the Aβ peptides form toxic assemblies, which initiate several processes leading to neuronal dysfunction and, eventually, large scale cell death.

In AD, the inflammatory component includes local astrogliosis, manifested as an increase in the number, size, and motility of astrocytes surrounding the senile plaques, and changes in microglia morphology, from ramified to amoeboid, accompanied by the expression and release of activation markers and pro-inflammatory mediators, including MHC class II, COX_2, MCP1, TNFα, IL1β, IL6, and prostaglandins like PGE2 (Akiyama, 2000). Interestingly, the chemokine MCP1 is known to induce the chemotaxis of astrocytes and contributes to the recruitment of these glial cells around senile plaques (Wyss-Coray, 2003). In addition, elevated levels of chemokines and cytokines, as well as their receptors, have been reported in post-mortem AD brains (Cartier, 2005).

Recently, specific studies have addressed the identification of the possible inducers or sensors of inflammation in AD. The inflammatory environment has been reported to be able to activate tau kinases, thus promoting the formation of NFTs (Ballatore, 2007), but whether hyper-phosphorylated tau and NFTs themselves contribute to the inflammatory response is not yet established.

Reactive hypertrophic astrocytes are intimately associated with the neuritic plaques

and surround amyloid aggregates with dense layers of thick processes. Significant activation of microglia appears at a very early stage of AD, before cognitive decline occurs (Vehmas, 2003). Throughout the progression of the disease, the chronic activation of local inflammatory cells and up regulation of pro-inflammatory mediators in response to the initial stimuli has been suggested to directly contribute to AD pathology or, else, significantly exacerbate the disease process (Cotman, 1996).

Although a pathogenic role for Aβ is generally accepted, the mechanisms remain poorly understood. Apart from the direct neuronal damage, Aβ aggregates have also been reported to promote microglia activation with the concomitant release of pro-inflammatory cytokines, chemokines, NO and ROS, which may contribute to neuronal death (Kitazawa, 2004).

Microglia and astrocytes seem to have a number of receptors, not fully characterized, capable to detect Aβ. Among them, the most relevant are TLRs (Landreth, 2009). These receptors activate the transcription factors NF-κB and AP-1, thus leading to the production of ROS and pro-inflammatory cytokines. In particular, Aβ has been reported to activate microglia and astrocytes through TLR_4, leading to a signal-dependent transcription factor cascade that drives the expression of downstream inflammatory response genes (Reed-Geaghan, 2009). Consistent with this, in 2008 Jin and colleagues showed that mice carrying a non-functional TLR_4 crossed with a mouse model of AD (APP/PS1 double transgenic mice) were characterized by a lower production of pro-inflammatory cytokines. However, given the fact that TLR_4 participates in the phagocytosis of Aβ plaques by microglia, and mice carrying mutant TLR_4 crossed with AD transgenic mice, have been reported to exhibit more Aβ plaques (Tahara, 2006).

TLR_2 is another example of TLR that might act as a sensor of Aβ in fibrils. Blocking TLR_2 signaling with specific antibodies or by in vitro receptor gene knock-down suggest that the Aβ–dependent stimulation triggers neurotoxic inflammation. In agreement with this finding, mice lacking TLR_2 crossed with APP/PS1 transgenic AD models were reported to have a delay in Aβ deposition and improved scores on memory tests (Richard, 2008).

On the whole, TLRs seems to have contradictory actions in vivo and in vitro, and this might reflect the complexity of the system, mainly due to heterogeneities in the cell signaling pathways that are engaged.

Another example of a receptor able to bind Aβ and to trigger an inflammatory response, is the receptor for advanced glycoxidation end-products (RAGE), a cell surface receptor belonging to the immunoglobulin superfamily (Schmidt, 2009). Several reports suggest that the Aβ peptides, as well as Aβ oligomers, bind to RAGE and activate glial cells, promoting the release of neurotoxic factors (Fang, 2010). This is confirmed by the fact that blocking the interaction of Aβ with RAGE inhibits the activation of microglia and reduces the production of pro-inflammatory mediators (Ramasamy, 2009).

A third described Aβ sensing system is represented by NOD-like receptors (NLRs), which are soluble, cytoplasmic pattern recognition receptors for pathogens, which also act as sensors of cellular damage. In AD, Aβ, either oligomeric or fibrillar, induces lysosomal damage and triggers NLR activation through $NALP_3$, a member of this receptor family mainly expressed by microglia (Halle, 2008). This binding leads to the activation of downstream proteins that results in the maturation and secretion of pro-inflammatory molecules.

Microglia can also play protective roles by mediating the clearance of Aβ through ApoE-dependent mechanisms or through the production and release of neurotrophic factors and/or anti-inflammatory mediators, such as TGFβ and PGE_2.

The relative roles of Aβ and other potential initiators of inflammation in AD remain unclear, but activation of glial cells in this context has been described to play a pivotal role in the production of numerous amplifiers of neuroinflammation. Cytokines like IL1β, TNFα or IL6, released from microglia, can directly act on neurons inducing apoptosis, or can trigger astrocyte activation. Apoptosis and necrosis of neurons result in release of ATP, which further activates microglia through the purinergic P_2X_7 receptor.

Factors released by astrocytes may lead to further activation of microglia. Moreover, pro-inflammatory cytokines are known to up-regulate the neuronal expression of APP, presenilins and the β-secretase BACE1, i.e. all the key proteins responsible for Aβ production (Sastre, 2008). Therefore, inflammatory mediators acting on neurons might contribute to enhance the production of Aβ that, in turn, activates microglia-mediated inflammation. This communication between neurons and glial cells probably leads to the amplification of the neurotoxic environment, contributing to AD pathology.

A final aspect is the increase in the number of microglia with a dysmorphic

appearance, typical of senescent cells, in postmortem AD brains. Streit and colleagues (2007) claimed that age-related telomere shortening, especially in locally replenished cell populations such as microglia, eventually renders cells senescent. They proposed that the development of AD is partly due to the loss of normal function in the senescent microglia, adding yet another element to the role of microglia in AD.

The strong inflammatory response involved in the pathogenesis of AD suggests that anti-inflammatory therapy would be beneficial in delaying the onset or slowing the progression of the disease. Epidemiological evidence suggests that anti-inflammatory agents such as non-steroidal anti-inflammatory drugs (NSAIDs) are associated with a reduced prevalence of AD (McGeer, 1996). However, data obtained from large randomized clinical trials using several different anti-inflammatory agents failed to prove an overall benefit. This discrepancy between the epidemiological data and the clinical trial data may, at least in part, be due to the inability of anti-inflammatory therapies to attenuate the detrimental effects of the chronic inflammatory state, which are already well established by the time clinical symptoms are first observed (Van Gool, 2003).

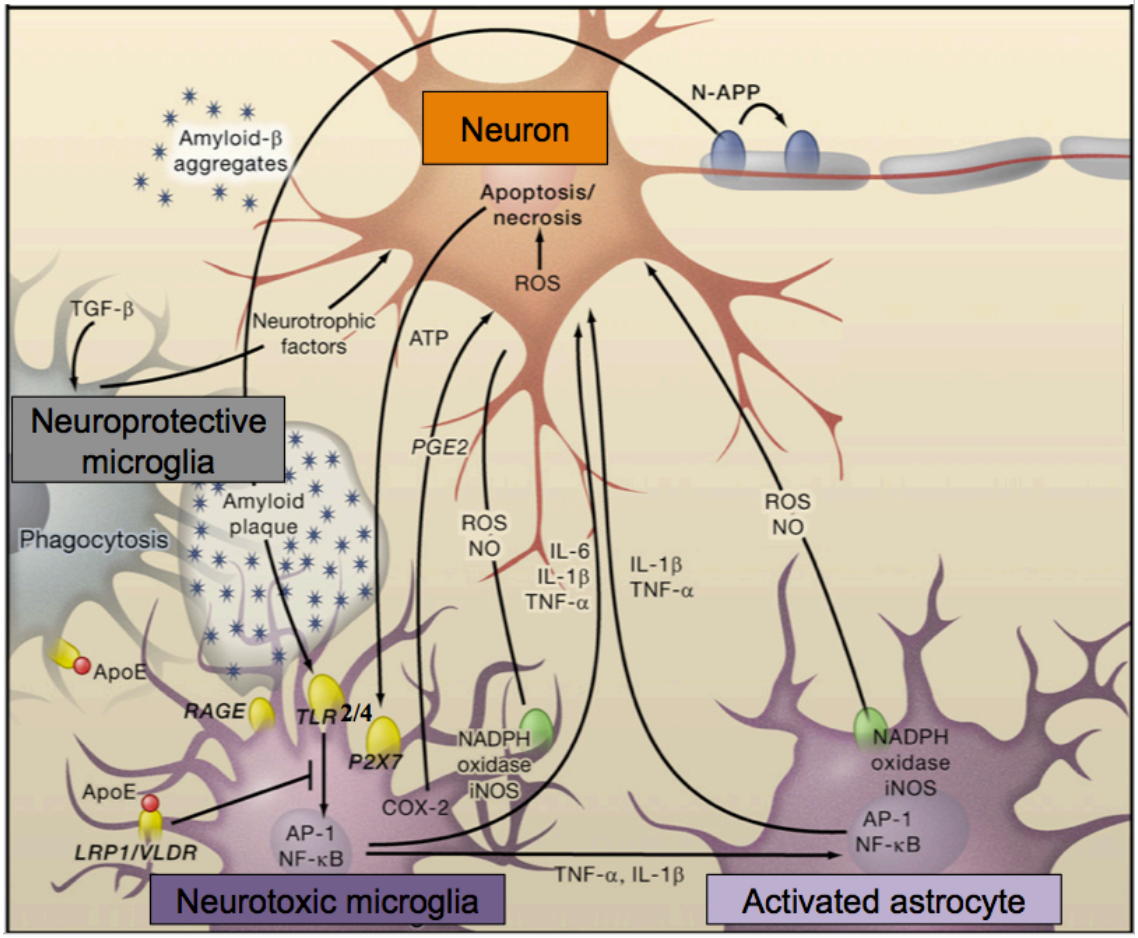


Fig. 1.10: Inflammatory components in Alzheimer's disease

Amyloid-β peptide, produced by cleavage of amyloid precursor protein (APP), forms aggregates that activate microglia through Toll-like receptors (TLRs) and RAGE. The consequent expression of NF-kB and AP-1 induces the production of reactive oxygen species (ROS) and inflammatory mediators such as cytokines. These inflammatory molecules act directly on neurons and also stimulate astrocytes, which amplify pro-inflammatory signals to induce neurotoxic effects. Apoptosis and necrosis of neurons result in release of ATP, which further activates microglia through the purinergic P2X7 receptor. Microglia can also play protective roles by mediating clearance of Aβ through ApoE-dependent mechanisms or through the production of anti-inflammatory and neurotrophic mediators that induce an inhibition of the inflammatory component by acting on the reduction of glia activation in both microglia and astrocytes. (Modified from Glass, 2010)

1.5.2 Multiple Sclerosis

Multiple sclerosis (MS) is a heterogeneous and complex autoimmune disease that is characterized by inflammation, demyelination, and axonal degeneration in the CNS. This pathology results from a primary defect in the immune system that targets components of the myelin sheath, resulting in secondary effects on neurons. The manifestations of MS include defects in sensation and in the motor, autonomic, visual, and cognitive systems (Hauser, 2006). In the early stage, approximately 85% of MS patients show the relapse-remission type of disease. However, with time, the recovery of these relapsing-remitting patients is impaired and eventually leads to an irreversible progression that is referred to as secondary progressive MS. The majority of relapsing-remitting MS patients progress to secondary progressive MS. However, about 10% of MS patients do not show any remission from the first attacks, and the primary neurological symptoms exhibit a continuous, so called, primary progression (Sospedra, 2005).

MS lesions are characterized by the infiltration of lymphocytes and antibody-producing plasma cells into the perivascular regions of the brain and the spinal cord white matter, along with an increase in microglia and astrocytes. The ensuing demyelination process (Lassman, 2001) is accompanied by deposition of antibodies and complement around the demyelinated lesions. Axonal degeneration can occur during the progression of the disease (Trapp, 2008).

Brain-imaging studies have clearly correlated the breakdown of the BBB and the CNS inflammation with the neurological disability characteristic of the initial clinical presentation and subsequent relapses of MS. Moreover, several aspects of the human disease pathogenesis have been clarified with the study of experimental animal models. The best MS animal model, i.e. the experimental allergic encephalomyelitis (EAE), has had a strong influence on shaping the current dogma of MS as a primary inflammatory demyelinating disease. In EAE models, rodents are immunized with a myelin-derived antigen and adjuvant, and, by combining the genetic background and the immunization protocol, EAE can reproduce the symptoms and the clinical courses of the human MS (Baxter, 2007).

Both innate and acquired immunity are involved in MS pathology. Outside the CNS,

immune cells like dendritic cells are recognized as key player of the disease (Bailey, 2007). The initial phase of MS is triggered by the innate system, although autoreactive T and B cells are present at a very early stage. After crossing the blood brain barrier, these cells proliferate and secrete pro-inflammatory cytokines that, in turn, stimulate microglia and astrocytes (Zamvill, 2003).

Microglia have been implicated in the initial stage of MS. This is supported by the evidence that, in the early phase of tissue damage, microglial activation is already present when the myelin sheath and the BBB, are still intact (Gay, 1997). Clusters of activated microglia expressing the human leukocyte antigen-DR (HLA-DR) and the microglial marker CD68, functioning as antigen presenting cells, are reported to be localized in pre-active MS lesions and are already evident in the absence of demyelination and leukocyte infiltration (De Groot, 2001).

In the active MS lesions, microglia are actively involved in the inflammatory response, by creating a favorable context for regeneration. Debris phagocytosis by microglial cells can promote clearance of the lesion and facilitate regeneration especially when T cells infiltrates are present (Nielsen, 2009).

While autoreactive T and B cells play roles as amplifiers and effectors in MS, microglial cells persist in active MS lesions in an activated state (Lassman, 2003), and produce cytokines like IL6, IL1β, TNFα, IL23, as well as ROS and NO, that, in turn, activate astrocytes and contribute to the neuroinflammatory context of the pathology. In the presence of IL6 and TGFβ, naive T cells are induced to express retinoic acid receptor-related orphan receptor γt (RORγt) and differentiate into Th17 cells, a subset of T helper cells that seems to have a crucial role in exacerbating EAE in mouse models (Cua, 2003). Activated microglia and astrocytes secrete IL23, which induce Th17 cells to secrete IL17 and TNFα (Korn, 2009) resulting in damage to the myelin sheath that protects nerve axons. Moreover, microglia may be stimulated to secrete toxic mediators after activation by an array of molecules including danger/stranger signals that bind innate receptors such as the TLRs, as discussed before, or through other pattern recognition receptors, such as scavenger receptors. Microglia may also be activated by complement and immunoglobulins that are present in MS lesions.

Cytokine and growth factors release from astrocytes has also been demonstrated in MS. In human MS tissue astrocytes were shown to express MHC class I (Höftberger,

2004) and class II (Zeinstra, 2000), although other studies showed absence of MHC class II expression in astrocytes (Bö, 1994). IL12, IL23 (Constantinescu, 2005) and IL17 (Tzartos, 2008) are also secreted by astrocytes, supporting their involvement in T cell presentation and activation.

From all these observations, it is possible to postulate that astrocytes are implicated in MS by expressing growth factors and cytokines, as well as by responding to such molecules in a paracrine manner. They take active part in the molecular concert that mediates the immune response and the lesion formation in MS; moreover, the balance of secreted molecules determines whether a toxic of protective phenotype will prevail.

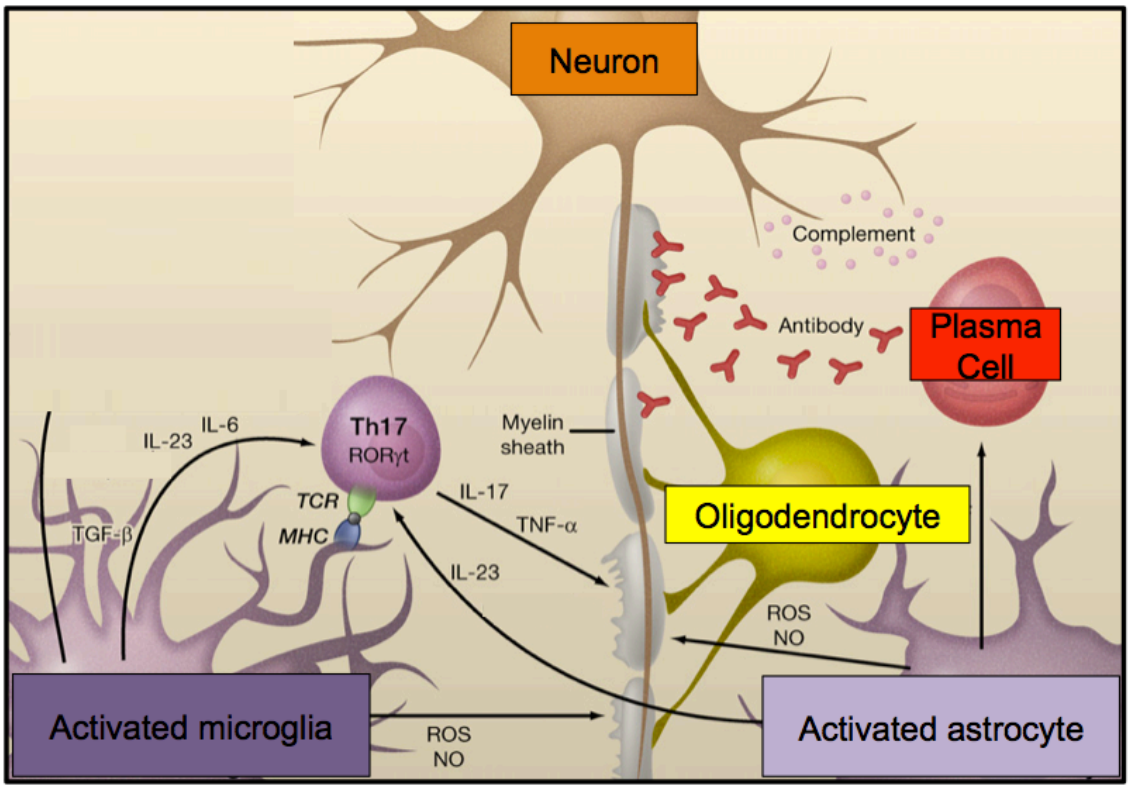


Fig. 1.11: Inflammatory components in Multiple Sclerosis

In MS an initial event determines activation of T and B cells with induction of immune responses against oligodendrocytes, the glial cells responsible for myelination of axons, and myelin components. After crossing the blood brain barrier, these autoreactive cells proliferate and secrete pro-inflammatory cytokines that, in turn, stimulate microglia and astrocytes. Activated glia cells release molecules that are sources of reactive oxygen species (ROS) and nitric oxide (NO), which contribute to the destruction of the myelin sheath and of the neurons themselves. They also release cytokines like IL6, IL23 and TGFβ, which induce naïve T cells to differentiate in Th17, thus promoting production of IL17 and TNFα resulting in damage of myelin sheaths. Finally, astrocyte activation promotes the release of mediators that induce autoreactive B cells to differentiate into plasma cells, resulting in the production of anti-myelin antibodies (Modified from Glass, 2010).

In conclusion, the role of microglia in MS, albeit important, is still largely unknown. The mechanisms leading to the various pathological patterns are complex and poorly understood. It has been hypothesized that unknown inflammatory events may cause a local release of inflammatory cytokines (TNFα, IL1β, and IL6) capable of triggering the production of secondary inflammatory mediators, such as chemokines, colony stimulating factors and lipid-derived molecules (Martino, 1999). Cytokines and chemokines would, in turn, allow the recruitment of leukocytes across the blood–brain barrier, including autoreactive T cells able to interact with resident antigen-presenting cells (Engelhardt, 2005), such as microglia and astrocytes. This local interplay is considered a crucial event leading to perpetuation and amplification of the inflammatory reaction, since the initial damage introduced by T cells is a stimulus for microglia activation and further recruitment of macrophages. Within this pathological framework, activated microglia and infiltrating macrophages are expected to produce a large number of harmful soluble factors, such as nitric oxide (Redford, 1997), inflammatory cytokines/chemokines (Muzio, 2007), excitotoxins (Smith, 2000), matrix metalloproteinases (Lindberg, 2004) and other proteases (Anthony, 1998). However, a view in which microglia may exert a reparative and anti-inflammatory action, with mechanisms still to be clarified, has recently emerged (Muzio, 2007).

Secretion of protective mediators, efficient phagocytosis of myelin debris and facilitation of remyelination are beneficial functions of microglia. Although many cytokines such as TNFα and INFγ are referred to as neurotoxic, it seems that their role is rather context-dependent. This would account for the fundamental importance of the microglia-mediated stimulation of growth and phagocytic removal of debris in creating a microenvironment for repair and regenerative processes.

In conclusion the elucidation of the protective mechanisms of microglia is of utmost importance to device new therapeutic strategies able to stimulate or potentiate the beneficial function of these glial cells.

2. AIMS OF THE WORK

Neuroinflammation is a CNS specific tissue response to injury. In physiological conditions there is a limited access of immune system to CNS. In response to a multitude of stimuli CNS can react in very different and complex ways.

In this heterogeneous context, the type of reaction of glial cells, astrocytes and microglia, acquires a pivotal role. Glia activation is a process occurring in CNS to maintain homeostasis in response to physiological or pathological signals, and chronic activation is a typical feature observed after brain injury or in neurodegenerative diseases.

The interplay of glial cells within the central nervous system is crucial to maintain the physiological function of the brain. It is known that specific stimuli can directly act on microglia cells inducing the release of pro-inflammatory mediators that, in turn, can activate astrocytes. Astrocytes activation leads to the modulation of gene expression, with increase in growth factors, cytokines, pro-inflammatory enzymes and cytoskeletal proteins. In physiological conditions it is known that astrocytes can contribute to restore the functional homeostasis by releasing signals that function as negative feedback on microglia activation. On the contrary, in pathological conditions they can amplify the inflammatory context. What is unknown and very debated in literature is whether all these effects, taken together, contribute to the detrimental or to the protective context of diseases. Based on these considerations, the aims of my work were:

- to study the process of glia activation by using molecules able to modulate the responses of microglia during the activation phases and to analyze the relative contributions of other glial cells with particular interest to the interplay between astrocytes and microglia.

- to characterize the activated phenotype of microglial cells in order to detect putative molecules functioning as specific markers, whose expression is modulated during the activation process.

The elucidation of these issues is important since it might help clarifying whether glia activation plays a beneficial or detrimental role towards the surrounding microenvironment, especially neurons, during neurodegenerative diseases. A comprehension of the mechanisms of the crosstalk between astrocytes and microglial cells, and a better characterization of microglial activated phenotype are expected to offer new perspectives for the development of therapeutic strategies for neurodegenerative disorders.

3. MATERIALS AND METHODS

3.1 Materials

Cell culture media and reagents were from BioWhittaker, Lonza group (Basel, Switzerland). Other chemicals, if not otherwise stated, were from Sigma-Aldrich St Louis, MO, USA). Culture flasks and multiwell plates were from Nalge Nunc (Rochester, NY, USA). Petri dishes were from Falcon BD (Franklin Lakes, NJ, USA).

3.2 Cell cultures

3.2.1 Primary cultures of pure rat cortical astrocytes

Primary cultures of cortical astrocytes were obtained from 1 to 2 day-old Sprague–Dawley rats (Charles River) according to McCarthy and De Vellis (1980).

Briefly, cortices were freshly dissected, cut into small sections with a razor blade and pieces were washed twice in Hank's Balanced Salt Solution (HBSS) supplemented with 10mM Hepes/Na pH 7,4, 12 mM $MgSO_4$, 50 U/ml Penicillin and 50 µg/ml Streptomycin (Gibco, Grand Island, NY, USA). Then, the tissue was dissociated with 2.5 mg/ml Trypsin type IX in presence of 1 mg/ml Deoxyribonuclease (DNase, Calbiochem, La Jolla, CA, USA) for 10 min at 37°C in two subsequent steps and the supernatants obtained were diluted 1:1 in medium containing 10% Horse Serum (PAA Laboratories GmbH, Pasching, Austria). The cell suspension was spun (100 g for 10 min) and cells were put in culture in Minimum Essential Medium Eagle (EMEM) supplemented with 10% Horse Serum (PAA Laboratories GmbH), 33 mM Glucose, 2 mM Glutamax (Gibco), 50 U/ml Penicillin, 50 µg/ml Streptomycin. Cells were maintained in 75 cm2 flasks (about 1 per pup) at 37°C in a humidified 5% CO_2 incubator. In order to remove microglia and oligodendrocyte progenitors and obtain pure cultures of type-1 astrocytes (> 99.5%), flasks were shaken at 200 rpm for 24 h at 37°C at days 2 and 6 after dissection, in Minimum Essential Medium with Hank's salts (HMEM) supplemented with 10% donor horse serum (PAA Laboratories GmbH), 33 mM glucose, 2 mM glutamax and 10 mM Hepes/ Na pH 7.4.

For biochemical and activation experiments cells were detached with buffered Trypsin (0.25%)/ Ethylenediaminetetraacetic Acid (EDTA, 1 mM) and re-plated with fresh medium on plastic multiwells (24 well plates; 100,000 cells per well) coated with poly-L-lysine (100 μg/ml, 5 min on the surface and then washed with H2O). Cells were used within 3 days after replating. Purity of astrocytic cells was assessed by morphological examination (immunofluorescence for glial fibrillary acidic protein, GFAP, and ionized calcium binding adaptor molecule 1, IBA1, markers for astrocytes and microglia, respectively) and the absence of response to LPS (in terms of upregulation of inducible nitric oxide synthase, iNOS, and secretion of IL6).

3.2.2 Rat microglia/astrocytes co-cultures preparation

Microglia cells were obtained from astrocytic flasks (as described above) by gentle manual shaking three days after dissection. Detached cells (about 80–90% microglia with a 20–10% astrocytic contamination) were plated on the top of pure astrocyte monolayers, making possible the preparation of co-cultures with known relative percentage of cells, or, alternatively, detached cells were plated in multiwells (150,000 cells per well in 24 well plates) coated with poly-L-Lysine (100 μg/ml) to obtain, within 2–3 days, astrocyte/microglia co-cultures with approximately a 1:1 ratio.

3.2.3 Rat highly enriched microglial cultures preparation

Highly enriched (>90%) microglial cultures were obtained by adding 25 ng/ml Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF; R&D Systems Minneapolis, MN, USA) to culture medium just after re-plating detached cells onto uncoated plastic multiwells. The resting state of unstimulated microglia was confirmed by the almost undetectable levels of IL6 secretion and iNOS expression.

3.3 Cell treatments

Primary cell cultures were treated with the following stimuli:

- Recombinant rat interleukin-1β (IL1β), TNFα and interferon γ (INFγ) were from R&D Systems, Minneapolis, MN, USA;
- LPS, isoproterenol, prostaglandin E2 (PGE2), sulprostone and butaprost were from Sigma-Aldrich, St Louis, MO, USA;
- rat α-CGRP (calcitonin gene-related peptide), CGRP8-37 (inhibitor of the CGRP receptor) and AM22-52 (inhibitor of the adrenomedullin receptors) were from Polypeptide Group (PolyPeptide Laboratories France SAS, Strasbourg);
- adrenomedullin (AM) was from Bachem, Torrance, California, USA;
- forskolin was from Calbiochem, La Jolla, CA, USA
- 8-bromoadenosine 3′, 5′-cyclic monophosphate (8Br-cAMP) was from Biaffin GmbH &Co KG, Kassel, Germany;
- Amyloid β peptide (1-42) was from American Peptide, Sunnyvale, California, USA.

Stock solutions of LPS, TNFα, IL1β, INFγ, CGRP, AM, CGRP8-37, AM22-52, isoproterenol and 8Br-cAMP were prepared in EMEM and stimuli administered with different dilutions to obtain concentrations described above. Forskolin, sulprostone, PGE_2 and butaprost were dissolved in dimethyl sulfoxide and administered with a 1:1000 dilution, i.e. a condition in which solvent alone was found to be ineffective on both basal activation of glia and LPS effects.

For assembly protocol, recombinant Aβ(1-42) peptide (American Peptide) was resuspended in dimethylsulphoxide (DMSO from Sigma-Aldrich) to 2.5 mM to obtain monomers, and diluted to 30 μM in EMEM red phenol-free. Oligomer and fibril preparations were aged for 24 h at 4°C and for one week at 37°C respectively.

Stimuli were administered directly to the culture medium as follows: rat cortical co-cultures astrocytes/microglia, were stimulated with IL1β (10 ng/ml), TNFα (30 ng/ml), INFγ (20 ng/ml), CGRP (100 nM), AM (100 nM), and LPS (10 ng/ml; Sigma-Aldrich cod. L2654), for 24 h at 37°C. Activated phenotype was tested by measuring the release of IL6, TNFα, and NO, and the upregulation of iNOS. Isoproterenol (1 μM), 8Br-cAMP

(100 μM), forskolin (10 μM), CGRP8–37 (3 μM) and AM22–52 (9 μM) were administered 30 min before pro-inflammatory stimuli administration.

3.4 Biochemical procedures and immunohistochemistry

3.4.1 Western blot analysis

Cells were washed twice with Phosphate Buffered Saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4) and lysed for 15 min at 4°C with 150 μl/well of lysis buffer (PBS supplemented with 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 10 mM EDTA/Na, and a cocktail of protease inhibitors, chimostatin, leupeptin, antipain, pepstatin, 10 μg/ml each). Lysates were centrifuged for 15 min at 15,000g at 4°C, the supernatants were collected and their total protein content analyzed by the MicroBCA reagent (ThermoFisher Scientific, Pierce, Waltham, MA, USA). About 20-40 μg of proteins were separated by standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE: 1.5 mm gel thickness) and then electrically transferred onto nitrocellulose membrane (0.45 μm pore size, Protran Whatman) in blotting buffer (2.5 mM TRIS, 19.2 mM glycine, 20% methanol). The membrane was stained with Ponceau S (0.2% in 3% trichloroacetic acid) for protein visualization and de-stained with bi-distilled water. After an overnight blocking in TBST (10 mM Tris/HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.6) containing 5% non-fat dry milk, at 4°C, membranes were incubated 2 h (room temperature, shaking) with 0.5 μg/ml of the primary antibody (mouse monoclonal anti iNOS antibody from BD Biosciences) and then, after an extensive washing with TBS containing 0.2% Tween-20, with horseradish peroxidase-conjugated anti-mouse secondary antibody (Bio-Rad). After three washing steps in TBST, protein signals were revealed on autoradiographic films (GE Healthcare, Piscataway, NJ, USA) by incubation with chemiluminescent solutions (Pico or Super Signal West Femto chemiluminescent kit, ThermoFisher Scientific, Pierce). ImageJ software (Collins, 2007) was used to perform the densitometric analysis of Ponceau S stained membranes in order to evaluate correct loading.

3.4.2 IL6 and TNFα determination

IL6 and TNFα were measured by sandwich enzyme-linked immunosorbent assay in 50 µl of supernatants collected from cocultures, according to manufacturer's instructions (Biotrak ELISA Systems, Amersham Biosciences, Uppsala, Sweden). In brief, IL6 and TNFα were measured from a non-diluted and a 1:20 diluted culture medium. With this system cytokines are immobilized to the bottom of an anti-rat specific antibody precoated plate, and a second biotinylated antibody is added, specific for a different epitope on the antigen. The signal is revealed by the binding of a streptavidin-HRP conjugate and the addition of a proper substrate solution. The quantification of the sample is obtained directly through the interpolation of the absorbance value at 450 nm in a cytokine standard curve. Limits of detection for IL6 and TNFα were 10 and 5 pg/ml, while limits of quantification were 40 and 15 pg/ml, respectively.

3.4.3 PGE_2 determination

For the detection of extracellular PGE_2, a competitive enzyme immunoassay (Prostaglandin E_2 EIA Kit-Monoclonal from Cayman, Ann Arbor, Michigan, USA) was used. Briefly, the method is based on the competition between PGE_2 present in cell supernatants and a PGE_2-acetylcholinesterase conjugate (PGE_2 tracer) for a limited amount of anti- PGE_2 monoclonal antibody. With this procedure, the quantity of PGE_2 tracer (kept at constant concentration) bound to the antibody is inversely proportional to the concentration of PGE_2 in the samples. Acetylcholinesterase substrate addition leads to the formation of a product that absorbs at 412 nm. The quantification was performed from 50 µl of a non-diluted and a 1:10 dilution of the medium recovered from control and activated astrocytes, according to manufacturer's instructions.

3.4.4 NO determination

NO production was determined by measuring the accumulation of nitrite in the culture medium. Nitrite was assayed colorimetrically by a diazotization reaction using the Griess reagent, composed by a 1:1 mixture of 1% sulfanilamide in 5% ortophosphoric acid and 0.1% naphtylenethylenediamine dihydrochloride in H_2O.
100 µl of culture medium were mixed to 100 µl of Griess reagent in a 96-multiwell plate and the O.D. at 550 nm was measured within 10 min. The nitrite concentration in the

samples was interpolated from a $NaNO_2$ standard curve ranging from 0 to 100 μM. The limits of detection and quantification were 0.25 and 0.7 μM, respectively.

3.4.5 Cell viability assay

Cell viability at the end of the treatments was assessed biochemically by standard 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and by fluorescence microscopy using sytox blue (Invitrogen) as dead-cell indicator.

Briefly, after treatment, pure cultures of astrocytes plated on 24-wells plates were incubated for 1 hour at 37°C with 0.5 mg/ml MTT in culture medium. After removing the extracellular solution, formazan, the MTT metabolic product, was dissolved in DMSO and the absorbance was read at 570 nm.

Changes in vitality, as well as total cellular protein content, upon treatments and among samples were less than 5%.

3.4.6 Immunofluorescence

Rat cortical pure astrocytes, co-cultures and highly microglia-enriched preparations, plated at subconfluent density on poly-lysine or not coated multiwell plates, were washed with PBS and fixed for 15 min with 3.7% paraformaldehyde in PBS at room temperature. After two washes with PBS, paraformaldehyde was blocked by two 5 min incubations with 50 mM NH_4Cl. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 min and incubated for 20 min with blocking solution (0.2% gelatin in PBS). The primary antibodies were diluted in blocking buffer and incubated for 30 min at room temperature. The following antibodies were used: polyclonal (Dako, 1:200 dilution) and monoclonal (Sigma-Aldrich, 1:200 dilution) anti GFAP antibody as astrocytic marker; polyclonal anti IBA1 (Wako Pure Chemical Industries, 1:200 dilution) as microglial marker; monoclonal anti iNOS (BD Biosciences, 1:100 dilution) as microglia activation marker. After three washes of 10 min with blocking solution, the secondary antibodies, either fluorescein isothiocyanate (FITC)- or rhodamine-conjugated goat anti mouse and anti rabbit immunoglobulin G (Invitrogen, both diluted 1:150 in blocking buffer), were incubated for 30 min at room temperature, in the dark. Multiwells were washed with blocking solution (three washes of 10 min), left in PBS, and observed by epifluorescence microscopy.

3.4.7 Isolectin-IB4 labeling

Highly microglia-enriched cultures were wash twice in PBS buffer and incubated 20 minutes at 4°C with isolectin-IB4 (Isolectin GS-IB4 from Griffonia simplicifolia, Alexa Fluor 568 conjugate, Invitrogen) diluted 1:1000 (from a stock solution in PBS at 1 mg/ml concentration) in EMEM with red-phenol. Then, cells were wash twice with cold PBS buffer and observed by epifluorescence microscopy.

3.4.8 RNA extraction and RT- qPCR

RNA was extracted from treated or untreated cells plated on 3.5 cm Petri dishes with TRIzol (Invitrogen) and phenol/chlorophorm/isoamyl alcohol (PCI, 25:24:1 v/v), following manufacturer instruction. Briefly, cells were lysed in 1 ml of TRIzol, to which were added 200 µl of PCI. After centrifugation (12,000 g, 15 min), the upper aqueous phase was transferred in a new tube and RNA was precipitated through addition of an equivalent amount of isopropanol. Samples were centrifuged (12,000 g, 10 min) and washed with 70% ethanol. RNA pellets were air-dried for 5 min, resuspended in 20 µl of RNase-free water and stored at −80°C.

Reverse transcription (RT) was carried out with random hexamers as primers, using Superscript III Retrotranscription Kit (Invitrogen) following manufacturer instruction. RT was carried on for 50 min at 50°C then stopped incubating samples at 85°C for 5 min. Single strand cDNA was obtained digesting complementary RNA strand with provided RNase H for 20 min at 37°C.

Quantitative polymerase chain reaction (PCR) was performed on a LightCycler 480 machine (Roche Diagnostics, Basel, Switzerland), with proprietary SybrGreen mix (LightCycler 480 Master Mix, Roche), following manufacturer instruction. Both forward and reverse primers were used at a 0.5 µM concentration. RT-derived cDNA was typically diluted at a 500 ng concentration before use. PCR program was performed with 10 min of denaturation step at 95°C and 35 to 45 cycles of amplification. Each cycle consisted of a denaturation step (95°C, 10 s), an annealing step (60°C, 25 s) and an elongation step (72°C, 15 s). After amplification, a melting step was performed (95°C for 30 s, 60°C for 1 min). Determination of Crossing points and Melting peaks was performed with LightCycler 480 Software (version 1.5.0.39, Roche).

Primers used (forward and reverse) were:

- gatccacattcggaggctaa and acgtgaaggttcaaggatgc for the gene encoding chemokine (CC motif) ligand 2 (CCL2, also known as monocyte chemotactic protein-1 or MCP1);
- ccaccgctgcccttgctgtt and cacccggctgggagcaaagg for the gene encoding chemokine (CC motif) ligand 3 (CCL3, also known as macrophage inflammatory protein-1α or MIP1α);
- acgagagccacaacgcagcc and tcaccccggatggaatggcct for the gene encoding interleukin 10 (IL10);
- gagcccagccacatcccgag and gtgcagcgcaccgttcttgc for the gene encoding CXC motif chemokine 10 (CXCL10, also known as interferon gamma-induced protein 10 kDa or IP10);
- gtatgaacagcgatgatgcact and gaagaccagagcagattttcaatag for the gene encoding IL6 (used as positive control for activation);
- gaagaagaaattagagaagcgttcc and gtagtttacctgaccatccccat for CALM2 (i.e. the gene coding for calmodulin 2, used as internal reference for normalization).

3.5 Transcriptomic analysis

Resting and activated astrocytes were subjected to RNA extraction using TRIzol (Invitrogen), as previously described. An amount of 500 ng of total RNA was reverse transcribed into cRNA (complementary RNA) and biotin-UTP labeled using the Illumina TotalPrep RNA Amplification Kit (Life Technologies) according to the manufacturer's protocol.

The gene expression profile was determined through the Illumina BeadArray technology using the RatRef-12 Expression Beadchips (Illumina Inc., San Diego, CA, USA). Each beadchip can process simultaneously 12 samples, each one investigated for more then 21,000 transcripts selected primarily from the NCBI RefSeq database (Release 16) and in a minor part from the UniGene database. 750 ng of cRNA were then hybridized to the BeadChip Array and stained with streptavidin-Cy3. All procedures were performed following the manufacturer's instructions. BeadChips have been imaged using the Illumina BeadArray Reader, a two-channel 0.8 μm resolution

confocal laser scanner and the Illumina BeadScan software. The software Illumina GenomeStudio v.2011.1 was used to elaborate the fluorescence signal to a value, whose intensity corresponds to the quantity of the respective transcript in the original sample. The same software was used to assess the system quality controls, such as biological specimen control, hybridization controls, signal generation controls and negative control. The samples have been tested in technical duplicates and a scatter plot with a correlation coefficient for each couple of replicates has been calculated. The mean correlation coefficient value obtained was 0.99.

3.6 Statistical analysis

Statistical analysis was performed with Prism software version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Columns in the graphs represent the mean (with SD or SEM) of at least three independent experiments performed in triplicate. Statistical significance was evaluated (with 95% confidence intervals) by unpaired two-tailed Student's t-test, for statistical analysis of two groups, or one-way ANOVA followed by Dunnett's (against the LPS treatment for multiple groups) or Bonferroni (for all pairwise comparisons) post hoc tests. A value of $p<0.05$ was considered to be statistically significant.

4. RESULTS

4.1 In vitro culture systems for glial cells

The interplay of glial cells within the central nervous system is crucial to maintain the physiological function of the brain. It is known that specific stimuli can directly act on microglia cells and induce the release of pro inflammatory mediators that, in turn, can activate astrocytes. In physiological conditions, astrocytes can contribute to restore the functional homeostasis by releasing signals that function as a feedback on microglia activation. However, in some pathological conditions, they can amplify the inflammatory context.

In the literature, the proportion and the role of microglial cells in astrocytic cultures are often underestimated. The main problem is that even small percentages of microglial cells can be responsible for effects observed on cultures in which the astrocyte is the most abundant cell type, thus making it difficult to spot the actual activated phenotype. If the relative contributions of astrocytes and microglia are not properly assessed, a microglia-derived effect may be erroneously attributed to astrocytes, just because they are the predominant cell type.

In this context, I established specific protocols of cell culture preparation in order to have controlled glial cultures. In particular, as described in details in the Materials and Methods chapter, I set up the conditions to have: i) really pure astrocytic cultures; ii) highly enriched microglial cultures; iii) an astrocytic monolayer co-cultured with controlled percentages of microglial cells; and iiii) astrocyte/microglia co-cultures with approximately a 1:1 ratio (Fig.4.1).

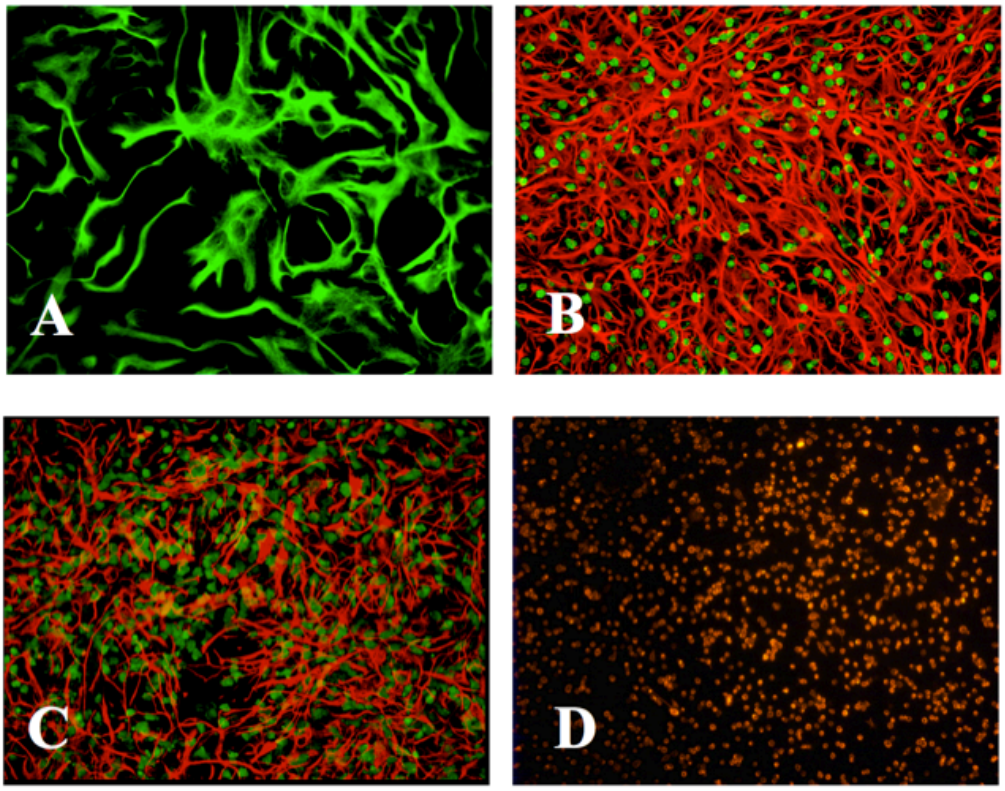


Fig. 4.1: Immunofluorescences of pure and mixed glial cultures

Pure astrocytic cultures (A), co-cultures astrocytes with 30% of microglial cells (B), and astrocytes/microglia 1:1 ratio (C) were all stained with anti-GFAP and anti-IBA1. In D enriched microglial cultures were stained by using the IB4 isolectin staining to assess the purity of the culture.

4.2 Characterization of an in vitro model of glia activation

In general, glia activation is characterized by multiple effects, such as induction of genes involved in the inflammatory process, release of biologically active molecules, morphological changes with concomitant modulation of cytoskeletal protein expression, and increase in the proliferation rate.

In order to study glia activation, I performed a characterization of this process by exposing the primary cultures of glial cells to pro-inflammatory stimuli. I firstly took in consideration the "purity" of the preparations and the fact that growing conditions in culture medium with horse serum instead of fetal calf serum, which is largely used in literature, were used to maintain a very low level of basal activation without interfering with the proliferation rate.

4.2.1 IL1β, TNFα and INFγ stimulation promotes the induction of the activated phenotype.

First of all, I started to characterize astrocyte activation by exposing primary pure cultures to a mix of pro-inflammatory cytokines that, in vivo, are described to be released by microglia as a consequence of activation. IL1β, TNFα and INFγ (10 ng/ml, 30 ng/ml, 20 ng/ml respectively) were added for 24 h to the culture medium and, then, astrocytes were analyzed for the induction of COX_2 and iNOS expression, as well as for the release of PGE_2, IL6 and NO, regarded as markers of activation.

When astrocytes were stimulated with the combination of IL1β and TNFα, a strong induction of the COX_2 protein was observed, in association with an increase of the release of PGE_2 and IL6. In this condition, expression of iNOS and NO release were barely detectable.

The addition of INFγ to the cytokine mix promoted an amplification of the enrollment of the above-mentioned markers and a strong induction of the expression of iNOS with concomitant NO release (Fig. 4.2).

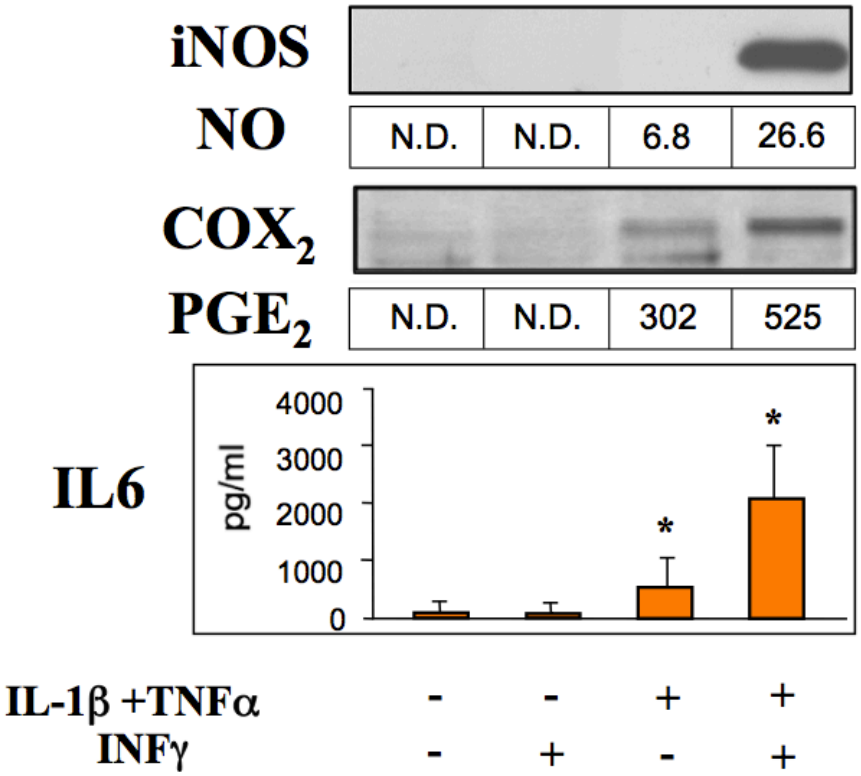


Fig.4.2: Induction of iNOS and COX2 proteins and release of IL6, PGE2 and NO in activated rat cortical astrocytes.

Western blot analysis was performed using anti-COX_2 and anti-iNOS antibodies, after 24 h incubation with INF γ (20 ng/ml) alone, IL1 β (10 ng/ml) + TNF α (30 ng/ml) or the combination of the three cytokines. Nitrite was quantified in the culture medium by the Griess reaction as a measure of NO release (μM Nitrite); PGE_2 and IL6 were quantified by ELISA (pg/ml). In the graph data are presented as means from three independent experiments. (ND=Not Detectable)

4.2.2 Effects of LPS on primary cultures

As previously mentioned, the importance of the purity of cells has been raised only recently in the literature. In particular, exposure to the lipopolysaccharide (LPS) from the gram-negative bacterial wall has been extensively used, as an alternative to pro-inflammatory cytokines, to activate astrocytes (Chen, 1998; Grimaldi, 1998; Kalmar, 2001).

In the experimental model proposed in my work, 24 hours of stimulation with high concentrations of LPS (i.e. 10 μg/ml) were ineffective in activating pure astrocytes. However, in presence of small contaminations of microglia (e.g. 0.5-1%), an increase of the activation markers could be observed. In fact, microglial cells respond to pathological stimuli by secreting biologically active molecules. Thus, the responses observed should be attributed to either direct microglia activation or astrocytes activation, as a secondary event, promoted by factors released by microglia.

In order to confirm this hypothesis, I administered LPS to either pure astrocytes or to "microglia-contaminated" cultures, derived from shaken or unshaken preparations, respectively, as described in materials and methods.

In pure astrocytes, LPS treatment failed, as said, to induce the activated state, while, in the presence of even small amounts of microglial cells (down to 0.5%), it was able to increase the expression of the pro-inflammatory markers (Fig.4.3).

I conclude that LPS cannot be used as an effective stimulus for astrocytic activation, unless in the presence of microglia. In any case, LPS challenge can be regarded as a very sensitive assay to reveal the presence of microglial contaminants in pure astrocyte preparations.

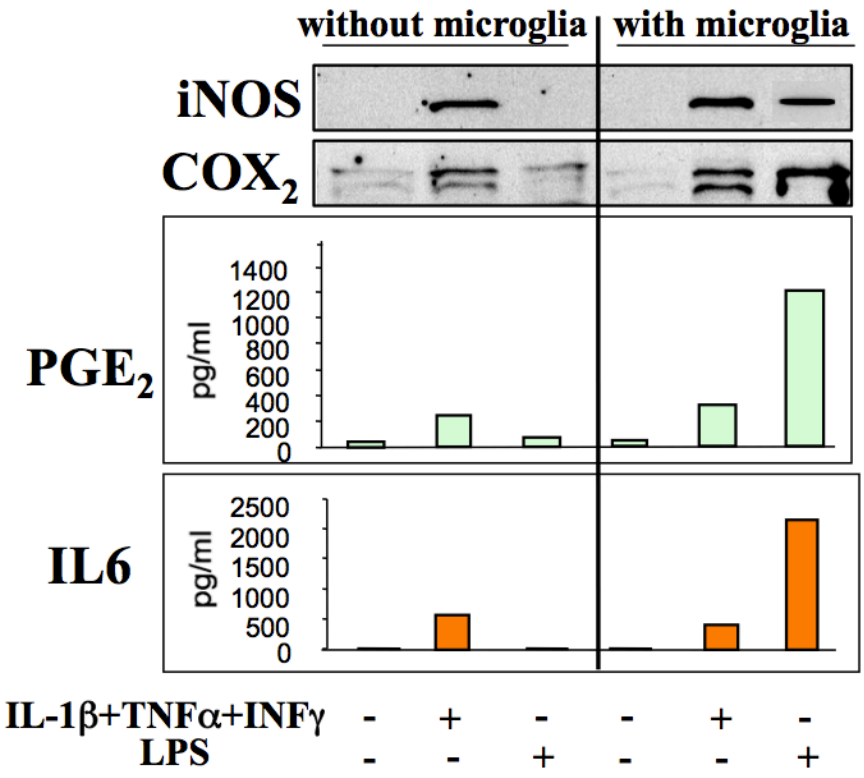


Fig.4.3: Effects of LPS treatment on the expression of markers of astrocyte activation in pure or "microglia-contaminated" astrocytic cultures.

Unshaken mixed glial cultures and pure astrocytic cultures obtained after 24 hours shaking to detach microglial and oligodendrocytic cells (see materials and methods) were treated with IL1β/TNFα/INFγ or LPS (10 μg/ml) for 24 hours. Culture medium was analysed for Il6 and PGE_2 by ELISA and cell lysates were analysed by Western blot to evaluate iNOS and COX_2 expression. LPS promotes astrocyte activation only in the presence of microglia, suggesting that LPS effect on astrocytes is indirect and mediated by microglia.

4.3 Amplification of inflammatory context during glia activation by amyloid β

Having established the conditions to keep primary culture preparations under control, and a well-characterized in vitro model of glial activation, I decided to investigate the possible modulations of glia activation.

4.3.1 Amyloid β and glial cells

Neuroinflammation refers to an inflammatory process that occurs in the CNS. It consists of a series of complex events that include microglial activation, astrogliosis, and the release of several inflammatory mediators.

Neuroinflammation has been assumed to be involved in almost all neurodegenerative diseases. However, due to its multifarious aspects, its precise role remains to be assessed.

The multifaceted nature of microglial activation is a theme that is present mainly in chronic disturbances of the CNS. As previously mentioned, in Alzheimer's disease (AD), multiple hypotheses have been put forward regarding the role of microglia.

A hallmark of AD is the presence of amyloid β (Aβ) aggregates. A finding that suggests a role for microglia in AD is the observation that soluble and oligomeric Aβ can activate microglia in culture (Maccioni, 2009). Microglia activated by soluble Aβ have a pro-inflammatory phenotype, upregulating TNFα and iNOS (Rao, 2006).

In mouse models of familial AD (plaque-generating mice strains commonly referred to as AD mice), genetically knocking down iNOS can alleviate amyloid plaque aggregation and premature mortality (Nathan, 2005). Treating AD mice with the anti-inflammatory drug minocycline also improves the clinical symptoms (Fan, 2007).

Several hypotheses have been postulated to explain the pathogenesis of AD, but none provides insight into the early events that trigger metabolic and cellular alterations in neuronal degeneration.

The amyloid hypothesis states that Aβ peptide deposition into senile plaques is responsible for neurodegeneration in AD. Recent findings also point to Aβ oligomers as the molecules responsible for the synaptic impairment that precedes neuronal degeneration. Another important pathological aspect is that cytokines and trophic factors can also have the anomalous effect of activating tau hyperphosphorylation. In

this context, a neuroimmunological approach to AD becomes relevant. When glial cells, which normally provide neurotrophic factors essential for neurogenesis and neuronal survival and repair, are activated by a set of stressing events, they overproduce cytokines and NGF, thus triggering altered signaling patterns that might be related to the etiopathogenesis of AD. A direct correlation has been established between the Aβ–induced neurodegeneration and cytokine production and release.

A variety of studies have documented, at least in vitro, the ability of Aβ fibrils to directly stimulate microglia that, as a consequence, could develop a neurotoxic phenotype characterized by the secretion of pro-inflammatory molecules. However, the direct effect of Aβ on glial cells is still unclear.

I decided to analyze the effects of Aβ on our primary cultures in order to test its putative role in the process of glia activation, in combination or not with other inflammatory mediators.

4.3.2 Effects of amyloid β on pure astrocytic cultures

One open question is whether reactive astrocytes around neuritic plaques can be activated directly by Aβ or they change phenotype only in response to the activation of microglial cells.

Evidence in the literature indicates that glial cells can be directly activated by Aβ and that this leads to the secretion of pro-inflammatory molecules. These studies focused their attention on rat astrocyte cultures (VonBernhardi, 2004; White, 2005), and they established that the stimulation with Aβ induces high levels of iNOS and NO, as well as an increase in IL1β.

In a first set of experiments, I started to analyze the effect of Aβ on pure astrocytic cultures. As represented in Fig. 4.4 (panels A and B), astrocytes, upon 24 hours stimulation with the mix of cytokines, acquired an activated phenotype, as expected. In order to obtain suitable Aβ preparations to stimulate the cells, the peptide underwent the treatments described in materials and methods to obtain the various steps of oligomerization. In particular, 10 μM monomeric Aβ or 5 μM of the oligomeric and the fibrillar forms of the peptide were used to stimulate astrocytes. LPS was applied to have a control of the presence of microglial cells. Aβ, in any form, was not able to trigger astrocyte activation, as demonstrated by the inability to promote NO and IL6 release, as

well as iNOS expression induction. Therefore, it can be concluded that Aβ alone is not able to directly induce the activation of astrocytes.

In order to study the possible influence of microglia, the same experiment was performed with unshaken rat primary astrocytes, i.e. cultures that contain 1 to 5% microglia. As illustrated in panels C and D, 24 hours 10 ng/ml LPS is effective in triggering the activated phenotype, thus confirming the presence of microglia in the astrocytic cultures. Surprisingly, in contrast to the data present in the literature, neither NO release nor iNOS expression was detected when cells were treated with the various forms of Aβ. Thereby, I concluded that Aβ alone did not promote glia activation.

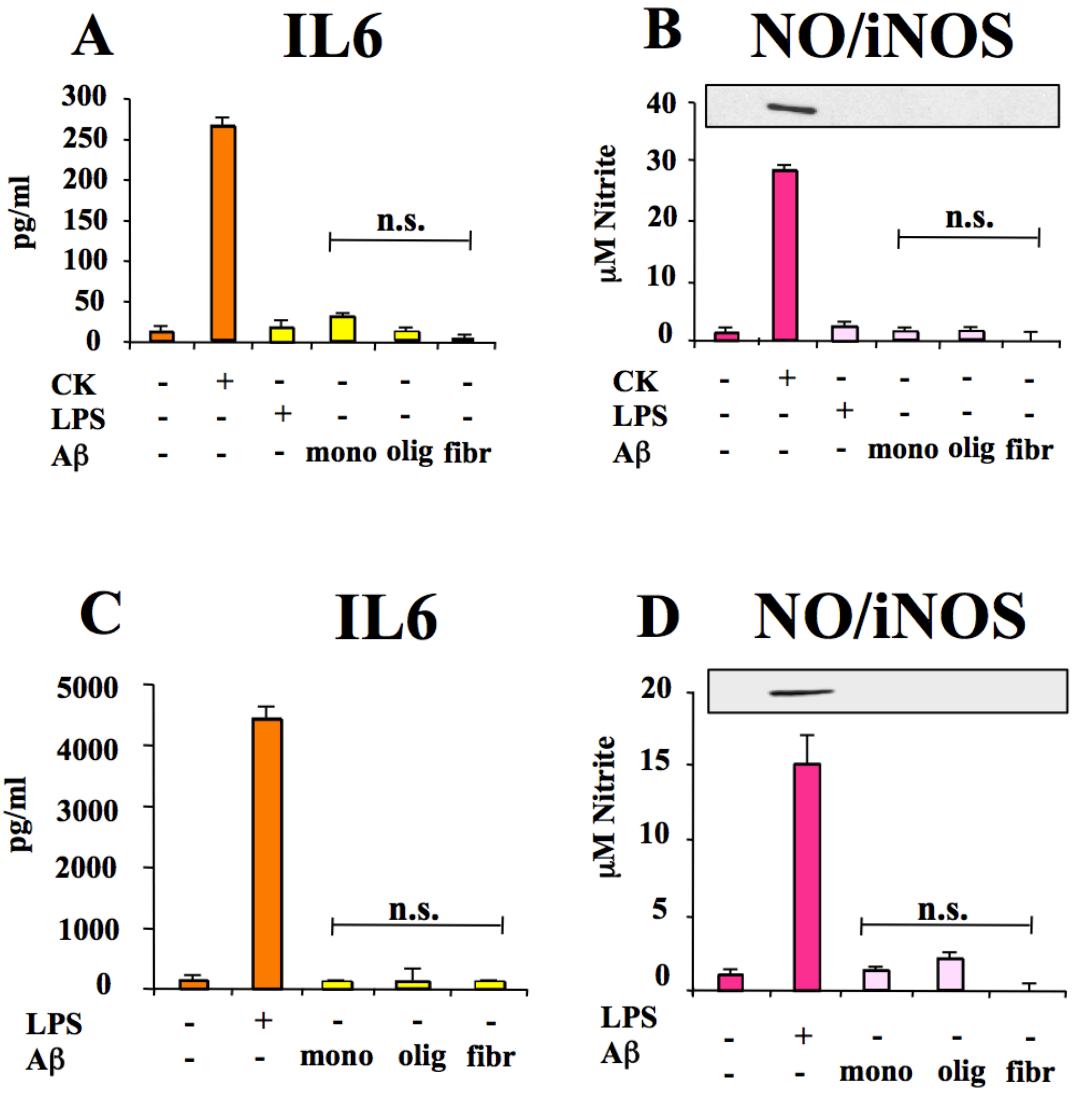


Fig.4.4 Aβ effects on astrocyte activation

Pure astrocytes (without microglial contamination) are stimulated with various forms of 5 μM Aβ for 24 h. LPS (10 mg/ml) is used as stimulus to check for the presence of microglial cells, while cytokine treatment (IL-1β, TNFα and INFγ) is used as positive control of astrocyte activation. Aβ alone is not able to directly activate astrocytes (A-B) even in presence of small contaminations of microglial cells (C-D). Statistical analysis (*p<0.05; **p<0.01; ***p<0.001): one-way ANOVA followed by Dunnett's post hoc test (against the controls).

4.3.3 Effects of amyloid β on co-cultures with precise percentage of microglia

Since small amounts of contaminating microglia have effects in promoting activation only with LPS, but not Aβ, I moved to study the effect of Aβ on mixed glial cultures.

More in detail, precise percentages (i.e <0.5%, 3%, 30%) of microglial cells were plated on a monolayer of pure astrocytes and tested upon stimulation with oligomeric and fibrillar Aβ or LPS.

Cells were incubated for 24 hours with 10 ng/ml LPS or 5 μM Aβ and then analyzed for the production of IL6 and the release of NO in association with the upregulation of iNOS. As illustrated in Fig. 4.5, the secretion and production of pro-inflammatory mediators, due to LPS stimulation, was strictly related to the percentage of microglial cells present in the culture, thus making the release of IL6 and NO, as well as iNOS expression, being stronger in cultures with the higher percentage of microglia. In the same way, a treatment with fibrillar Aβ had a similar trend in relation to the presence of microglial cells, but the values in terms of production of pro-inflammatory factors were much lower. According to this result, I can conclude that fibrillar Aβ induces activation of microglia with an efficiency (and, possibly, a mechanism) that seems not to be the same as LPS. Conversely, the oligomeric form of Aβ was ineffective in activating the culture, even in the presence of 30% microglia.

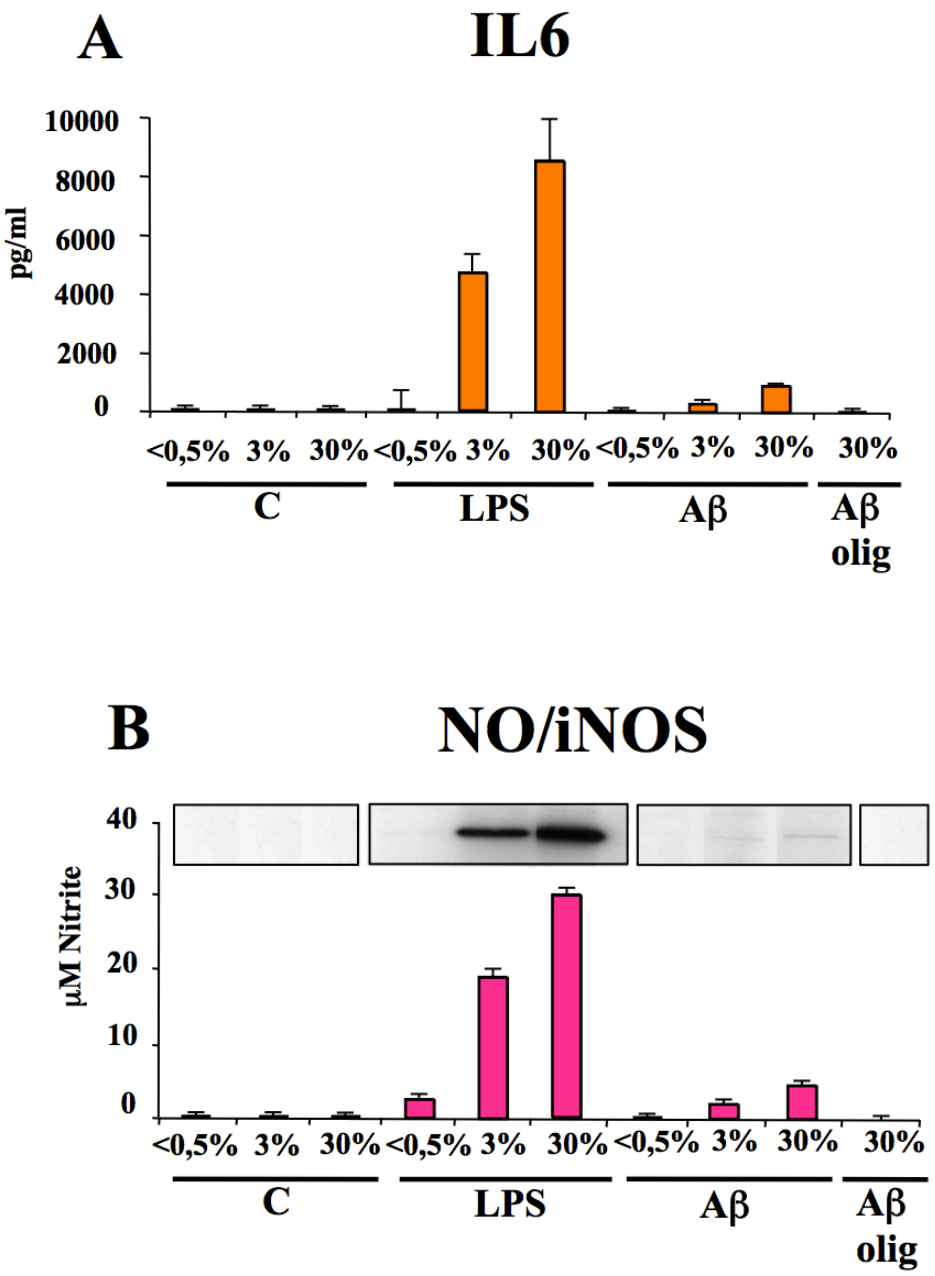


Fig. 4.5: Fibrillar amyloid β activates microglial cells

Rat cortical astrocytes with different amounts of microglia (<0,5-3-30%) were stimulated with 5 µM fibrillar Aβ for 24 h. LPS is used as marker of the presence of microglial cells and it is used as positive control of activation. Higher amounts of microglial cells on astrocytic cultures (3-30%) promote iNOS expression following fibrillar Aβ treatment. Also the increase in IL6 release is related to the proportion of microglial cells.

4.3.4 Effects of amyloid β on highly enriched microglial cultures.

Taking into consideration that Aβ has a role in microglia activation in glial co-cultures with increasing amounts of microglial cells, I next studied the effects of this peptide, in its oligomeric and fibrillar forms, by employing highly enriched microglial cultures.

In this set of experiments, cells (>90% microglial cells) were treated with 5 μM Aβ for 24 hours, and the putative activated phenotype wasrecognized by analysis of IL6 and NO release, as well as iNOS expression. LPS (10 ng/ml) was used as a positive control for activation. As shown in Fig. 4.6, looking at the pro-inflammatory markers, both oligomeric and fibrillar Aβ failed to promote a clear activated phenotype.

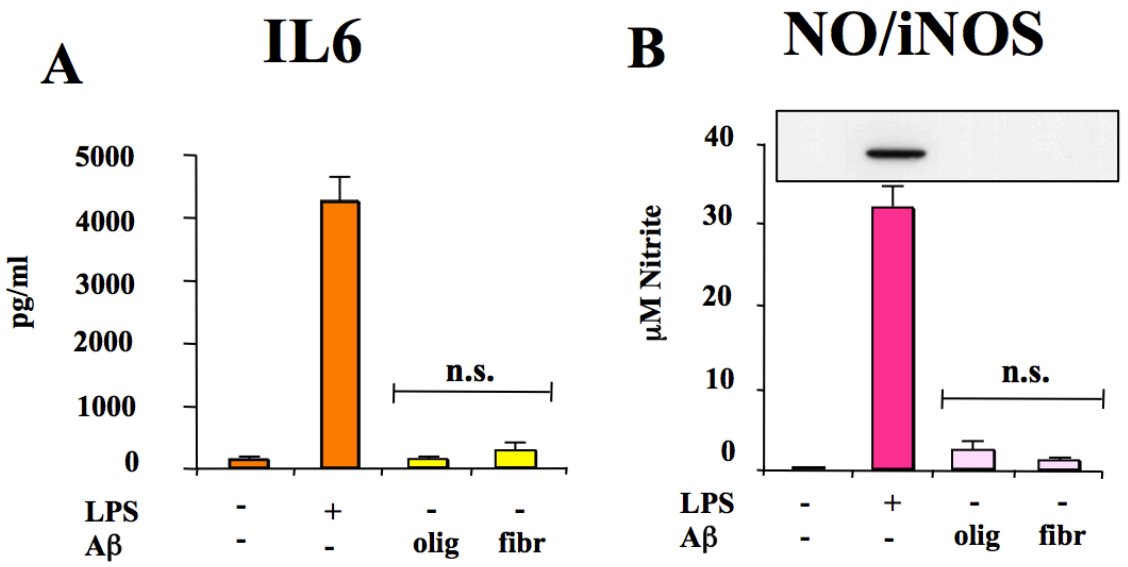


Fig. 4.6: Effects of amyloid β on enriched microglial cultures

Highly enriched microglial cultures were treated with 10 ng/ml LPS and 5 μM oligomeric/fibrillar Aβ, and the activated phenotype was evaluated via NO and IL6 release as well as iNOS expression.

No induction of pro-inflammatory markers was found upon Aβ administration, while LPS, as usual, triggered the activated phenotype. Statistical analysis (*p<0.05; **p<0.01; ***p<0.001): one-way ANOVA followed by Dunnett's post hoc test (against the controls).

4.3.5 Effects of amyloid β on LPS stimulation on glial cultures

As already mentioned, the interplay between astrocytes and microglia is a crucial aspect when studying the effects of the various activating stimuli. In order to investigate the contribution of amyloid β in an inflammatory context, I performed stimulations on both astrocytes/microglia co-cultures and enriched microglia cultures with LPS in the presence of fibrillar Aβ. In particular, cells were stimulated with 10 ng/ml LPS and 5 μM Aβ for 24 hours, and then supernatants and cell lysates were analyzed.

As represented in Fig 4.7, when Aβ was added in combination with LPS, I found an amplification of LPS effects, in terms of IL6 and NO release, as well as iNOS induction (A-B). The enhancement of IL6 production is also appreciable in highly enriched microglia cultures, but it was not as strong when considering NO release and iNOS expression (C-D). This kind of synergy ("superactivation") that occurs mainly in the co-culture system, underlines the importance of astrocyte in the modulation of glia activation.

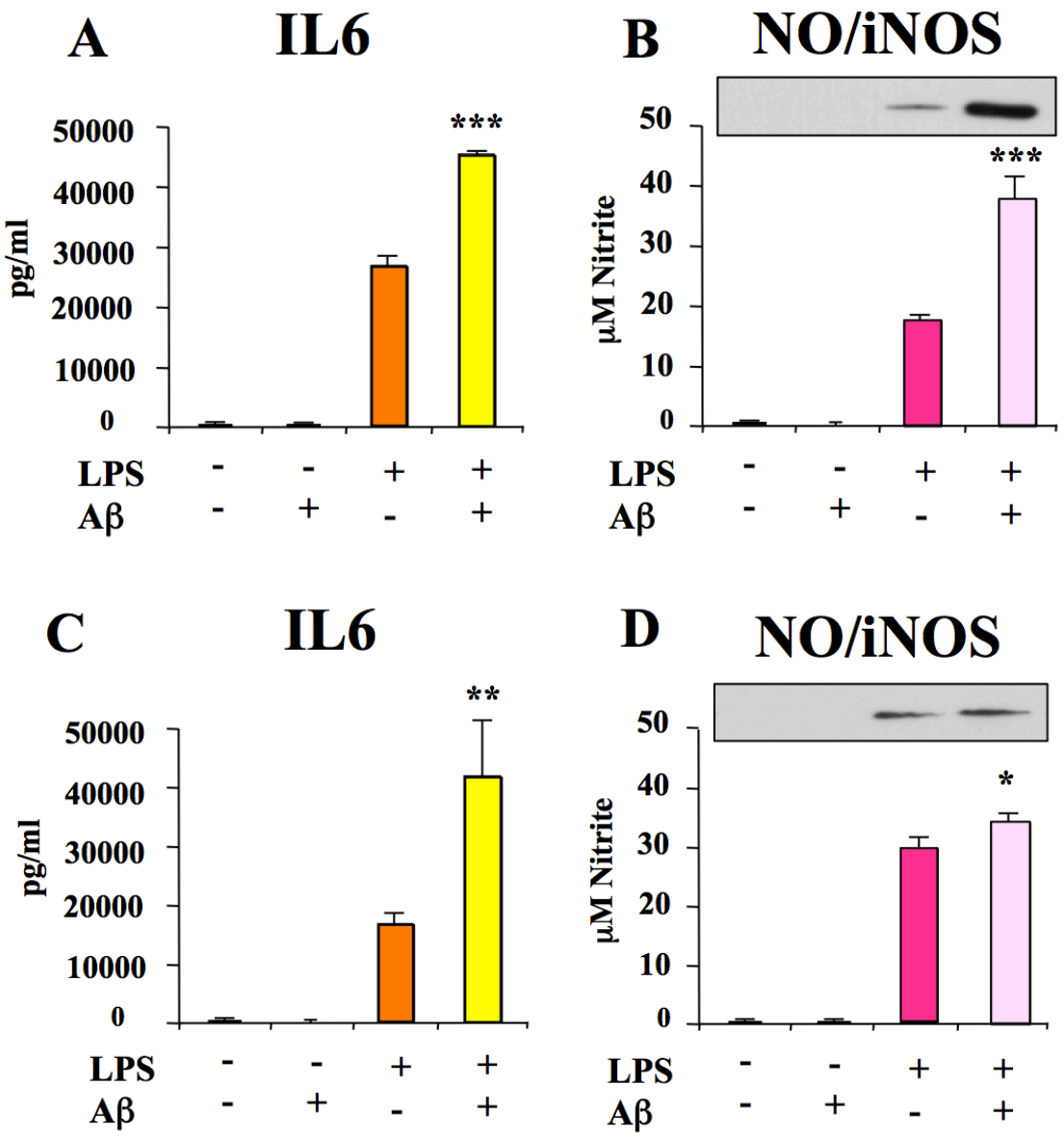


Fig. 4.7: Effects of amyloid β on LPS stimulation.

Co-cultures of astrocytes/microglia in a ratio of 1:1 (A-B) and highly enriched microglial cultures (C-D) were treated for 24 hours with 10 ng/ml LPS alone or in combination with 5 µM Aβ in its fibrillar form. In co-cultures LPS, as expected triggered the activated phenotype and the concomitance administration of Aβ was able to amplify IL6 and NO release as well as iNOS expression. This increase in production of pro-inflammatory markers was observed also in enriched microglial cultures in terms of IL6 (C) secretion but it was clearly lower considering NO release and iNOS expression (D). Statistical analysis (*$p<0.05$; **$p<0.01$; ***$p<0.001$) was calculated using one-way ANOVA followed by Dunnett's post hoc test (against LPS treatment).

4.4 Negative modulation of glia activation

As described in the introduction, during the inflammatory response, further damages may arise from potential autoimmune responses occurring when the immune cells react to self-antigens. Accordingly, the identification of endogenous factors that can keep under control an exacerbated immune response is a key goal for the development of new therapeutic approaches for inflammatory and autoimmune diseases. In particular, some neuropeptides, produced during an ongoing inflammatory response, have emerged as endogenous anti-inflammatory agents that might tune the state of the immune system. These neuropeptides participate in maintaining immune tolerance through two distinct mechanisms: by regulating the balance between pro-inflammatory and anti-inflammatory factors, and by inducing the emergence of regulatory T cells with suppressive activity against autoreactive T cell effectors. In the following part of my work, I focused the attention on the role of two specific neuropeptides, Calcitonin Gene-Related Peptide (CGRP) and adrenomedullin (AM), in the inflammatory context.

4.4.1 Effects of CGRP and AM on glial cells

CGRP and AM belong to the CGRP/calcitonin peptide superfamily (Amara, 1982; van Rossum, 1997). Their receptors, in order to be active, require the association of the calcitonin-like receptor (CLR) with the Receptor Activity-Modifying Proteins 1–3 (RAMP1–3) (McLatchie, 1998). The association with the different members of the RAMP family confers these receptors the specificity for the various peptides of the family (Born, 2002): CLR/RAMP1 complex forms the CGRP receptor (antagonized by the CGRP antagonist CGRP8–37), while CLR assembly with RAMP2 and RAMP3 gives rise to the AM receptors 1 and 2, respectively (Poyner, 2002). The interaction of these receptor complexes with an additional cytoplasmic protein, the Receptor Component Protein (RCP) is required for the activation of signal transduction cascades, including cyclic adenosine monophosphate (cAMP) formation (Evans, 2000; Luebke, 1996).

CGRP/AM receptors are widespread in endothelial, vascular smooth muscle, immune, glial and neuronal cells (Hay, 2004). One of the most studied and powerful

physiological effects elicited by CGRP/AM receptor activation is vasodilation (Brain, 2004). In brain, the potent and long-lasting activity of CGRP on arteries and veins modulates local blood flow and extravasation, and is thought to be involved in headache (Geppetti, 2005). Also AM, which is produced by endothelial and vascular smooth muscle cells, has been proposed to play a role in the regulation of systemic blood pressure. In particular, AM inhibits endothelial cell contraction and junctional disassembly, thereby limiting vascular permeability and edema during inflammation (Temmesfeld-Wollbruck, 2007).

Among the various effects of CGRP and AM, their ability to modulate the immune/neural-immune system is of utmost interest and the emerging picture indicates that the two peptides can exert pro- as well as anti-inflammatory actions in a cell/tissue-specific and stimulus-specific manner. For instance, experiments on animal models demonstrate anti-inflammatory effects of CGRP (Gomes, 2005; Kroeger, 2009; Tsujikawa, 2007) that set against the neurogenic inflammatory action widely reported in periphery and brain (Durham, 2010). Similarly, both pro-inflammatory and anti-inflammatory roles have been proposed for AM (Dackor, 2007; Ma, 2010; Miksa, 2007) although very little is known about its activity in the central nervous system.

Based on these assumptions, CGRP and AM are expected to be involved in several neuroinflammatory conditions and to play an important role in some neurodegenerative processes. For instance, the inflammatory component of multiple sclerosis is characterized by a complex interplay of cells (resident microglia, astrocytes, infiltrating macrophages and T cells), mediated by released molecules, such as tumor necrosis factor α (TNFα), interleukin-6 (IL6) and nitric oxide (NO), that can exert a detrimental role on neuronal function (Encinas, 2005; Hartung, 1995; Martino, 2000). Moreover, chemokines released by vascular and perivascular resident glial cells are known to favor infiltration of lymphocytes into the parenchyma of the central nervous system, an obligatory step for the progression of the autoimmune attack (Engelhardt, 2005; Szczucinski, 2007).

Here I investigated the effects of CGRP and AM in glial cultures, showing that CGRP and AM exert a potent and efficient anti-inflammatory role on microglia activation by inhibiting the lipopolysaccharide (LPS) induced release of pro-inflammatory molecules.

This previously unrecognized role of CGRP and AM on glial cells might be relevant in the neurodegenerative processes by inhibiting the inflammatory process and stimulating repair events. It follows that these findings may represent a new paradigm to devise therapeutic strategies for neuroinflammatory diseases, such as multiple sclerosis.

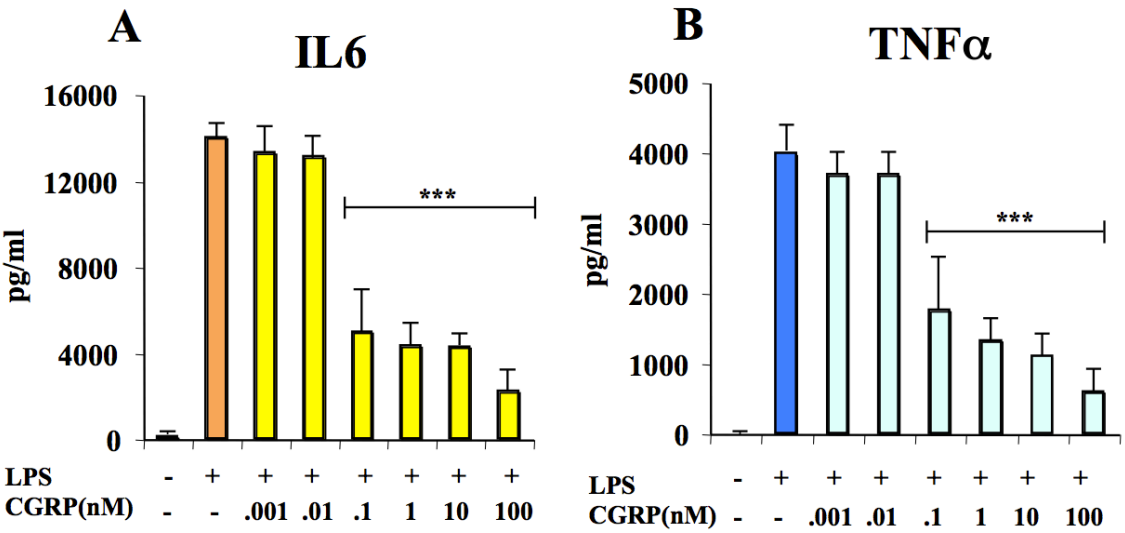
4.4.2 CGRP and AM inhibit LPS-induced glia activation

I first evaluated the effects of CGRP and AM on glia activation, by stimulating microglia and astrocytes in culture. I started to analyze the possible interaction between these two kind of glial cells, thus exposing microglia seeded on a layer of pure astrocytes (co-cultures ratio 1:1, as described in materials and methods chapter) to different treatments.

As expected from previous data, 24 hours exposure to 10 ng/ml LPS induced a marked increase in IL6 secretion, TNFα release, iNOS expression and NO production. These data confirm that LPS is capable to promote the expression and the secretion of the most common markers of glia activation by directly acting on microglia cells, as previously commented, that may in turn trigger astrocyte activation.

When I applied CGRP in combination with LPS in these co-cultures, I observed a dose-dependent inhibition of activation, as illustrated in Fig. 4.8.

The same effects as with CGRP were observed by stimulating co-cultures with AM in combination with LPS. The same kind of dose-dependent inhibition on LPS treatment was appreciated, as an explanation for the potent anti-inflammatory effect of both neuropeptides.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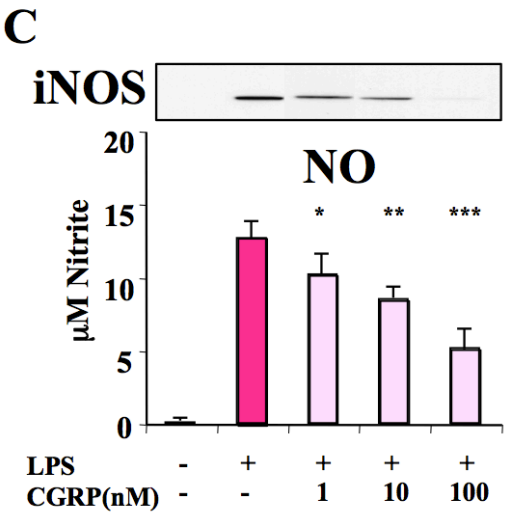
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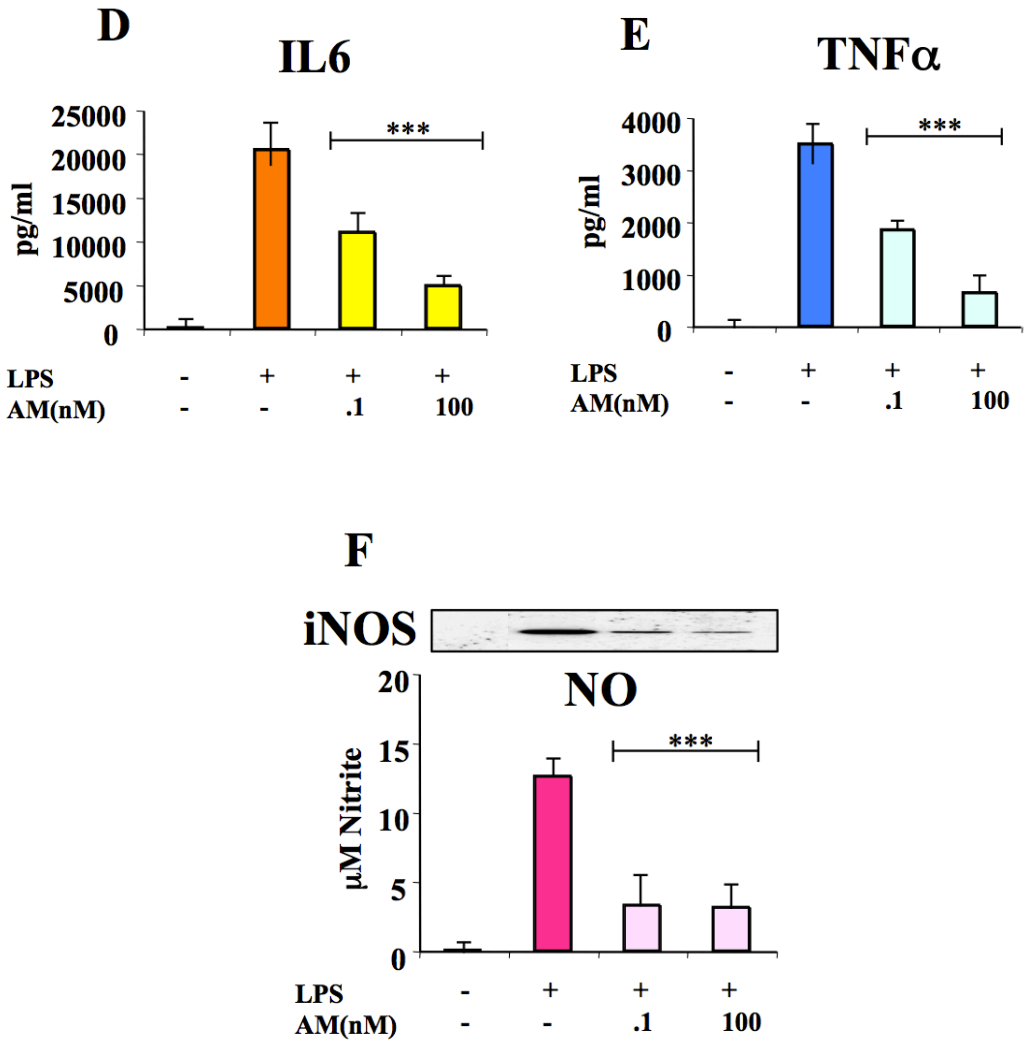
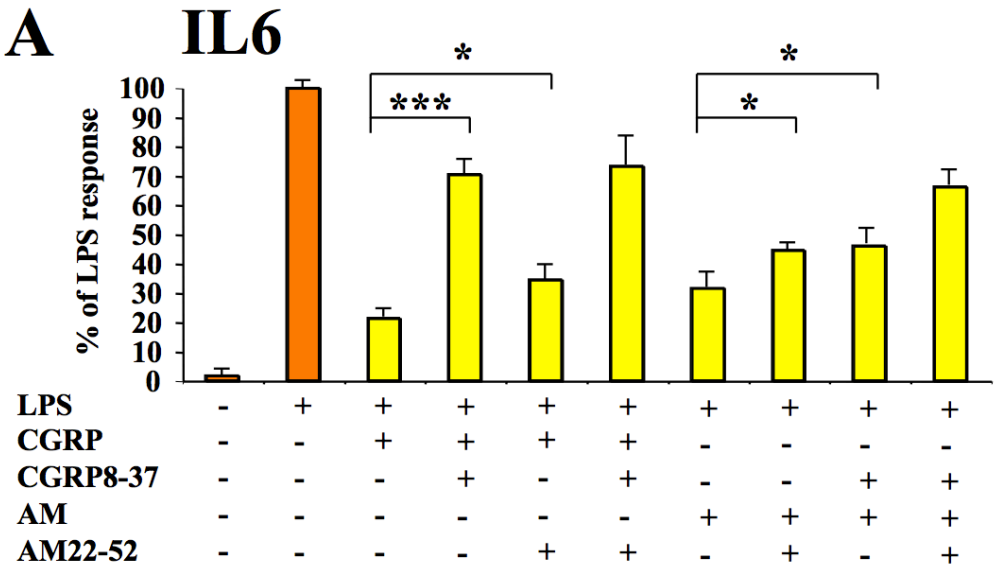


Fig.4.8: CGRP and AM inhibit LPS-induced activation in microglia/astrocyte co-cultures. Microglia/astrocyte co-cultures were treated for 24 h with LPS (10 ng/ml) and increasing concentrations of CGRP (A, B, and C) or AM (D, E, and F). At the end of the treatment the medium was tested for the release of IL6 (A and D) TNFα (B and E) and NO (C and F). Inset in (C and F) shows the corresponding western blot for iNOS performed on cell lysates. In this and the following figures, the columns in the graphs represent the average (+SEM) of at least three independent experiments performed in triplicate. Statistical significance (*p<0.05; **p<0.01; ***p<0.001) was calculated using one-way ANOVA followed by Dunnett's post hoc test (against the LPS treatment).

I then used two antagonists of the CGRP and AM receptors (CGRP8-37 and AM22-52, respectively), in order to verify the pharmacological profile of the effects of the neuropeptides. More in detail, I preincubated co-cultures with 3 μM CGRP8-37 and 9 μM AM22-52, by adding them on culture medium 30 minutes before pro-inflammatory and/or CGRP/AM stimulation.

As illustrated in Fig. 4.9, the two antagonists partially reduced the inhibitory action of CGRP and AM on co-cultures treated with LPS, as revealed on IL6 secretion, TNFα release and NO production. This result is in line with the notion that microglial cells are potentially able to form all the receptors for CGRP and AM (Moreno, 2002), but also reveals the different extent of inhibition exerted by these two antagonists. This could reflect the ligand-receptor promiscuity of this system, in which CGRP may also act on AM receptor 2 and AM is also able to activate the CGRP receptor (Poyner, 2002). On the other hand, it must be considered that AM22-52 acts with different potency on the two AM receptors (Hay, 2004).



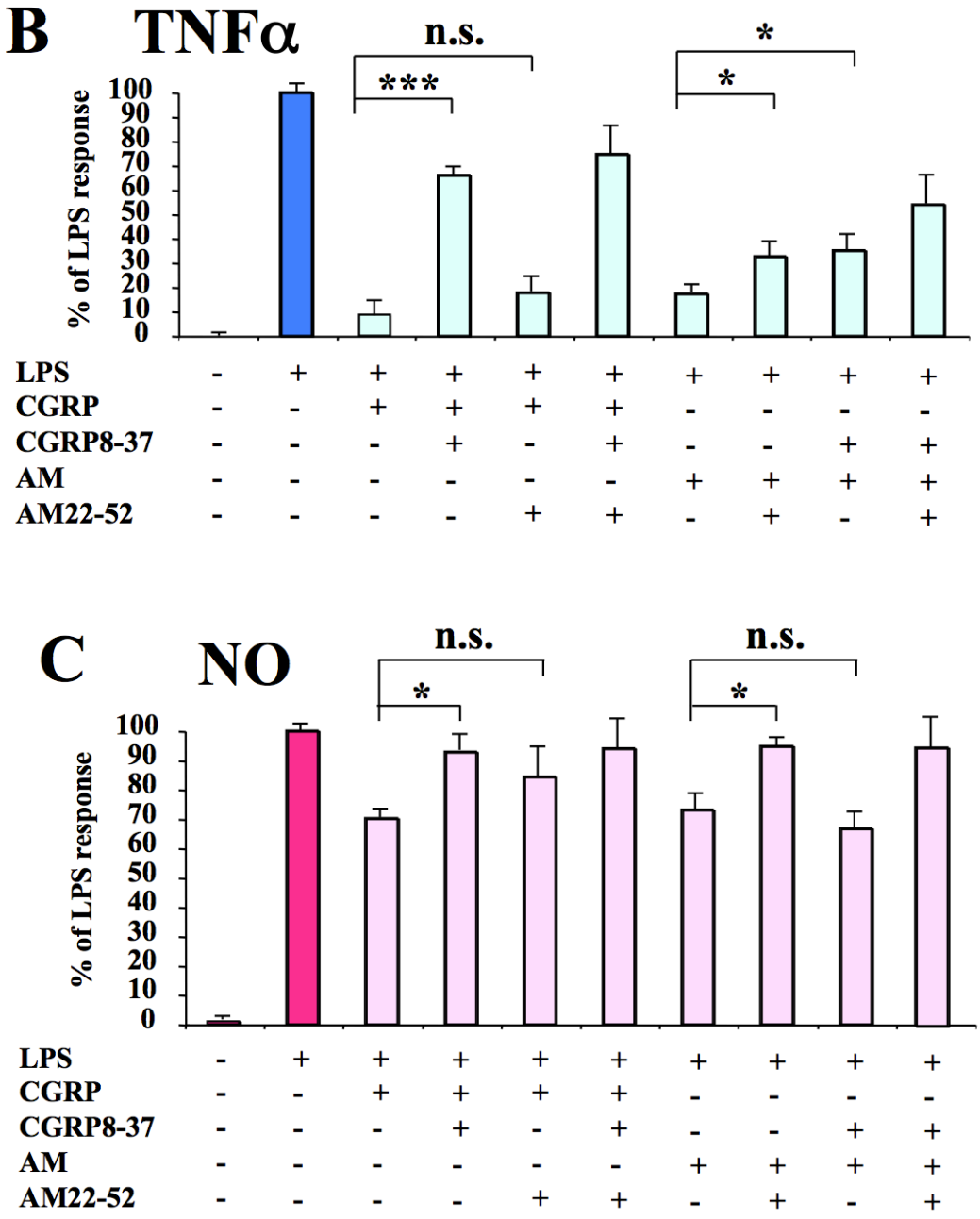


Fig. 4.9: CGRP8–37 and AM22–52 partially revert the effects of CGRP and AM.

Microglia/astrocyte co-cultures were activated for 24 h with LPS (10 ng/ml) and various combinations of agonists (CGRP or AM, 10 nM each) and antagonists (CGRP8–37 or AM22–52, 3 µM and 9 µM, respectively) of CGRP and AM receptors. At the end of the treatment the medium was tested for the release of IL6 (A) TNFα (B) and NO (C). Statistical significance (*p<0.05; **p<0.01; ***p<0.001) was calculated using one-way ANOVA followed by Bonferroni post hoc test.

To verify whether the results obtained in the co-cultures were influenced by the interplay between the two cell types, I performed experiments on highly enriched microglia cultures (~95% purity).

LPS was again able to increase the three markers of activation, although with a different pattern of release. In fact, in microglia cultures the ratio between TNFα and IL6 was reduced (about 3–4 folds) with respect to co-cultures suggesting that astrocytes significantly contribute to the release of cytokines with a distinct pattern.

In this experimental cellular model as well, CGRP and AM exerted an inhibitory effect on the induction of LPS activation (Fig.4.10).

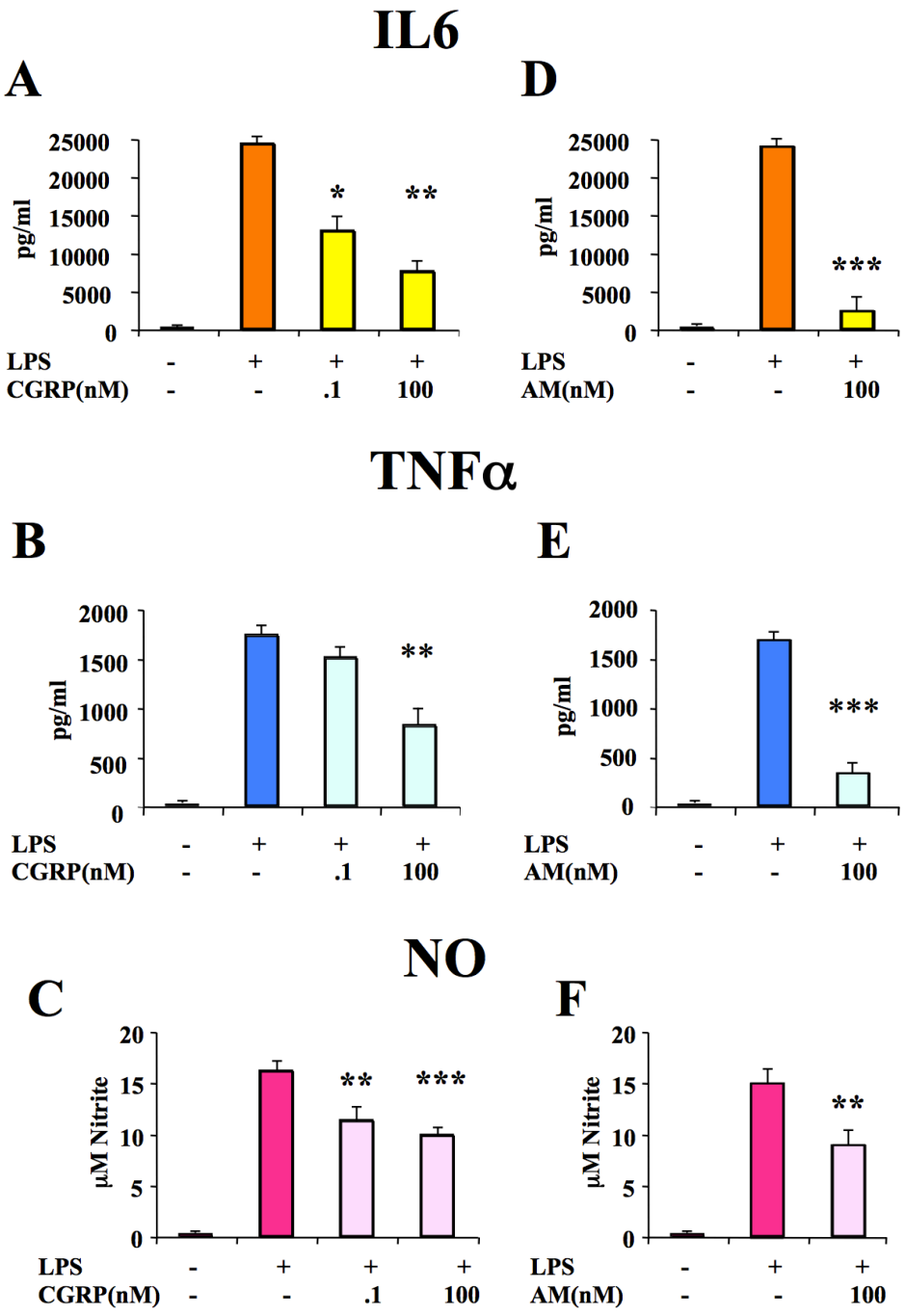


Fig. 4.10: CGRP and AM inhibit LPS-induced activation in microglia-enriched culture. LPS (10 ng/ml) was given to preparations of primary microglia cells for 24 h in the presence or absence of CGRP (100 nM) or AM (100 nM). Secretion of interleukin-6 (A and D) tumor necrosis factor α (B and E) and NO (C and F) was then tested in the cell supernatant. Statistical significance (*p<0.05; **p<0.01; ***p<0.001) was calculated using one-way ANOVA followed by Dunnett's post hoc test (against LPS treatment).

4.4.3 CGRP and AM do not inhibit glia activation promoted by cytokine administration

Since also pro-inflammatory cytokines have been reported to induce glia activation, I evaluated the effects of the two neuropeptides upon exposure of cells to the previously employed mix of cytokines (IL1β 10 ng/ml, TNFα 30 ng/ml, and INFγ 20 ng/ml).

Microglia/astrocytes co-cultures, pure astrocytes and highly-enriched microglia were analysed for their activated phenotype upon stimulation with various combinations of with both LPS, cytokines and the two neuropeptides, CGRP and AM. In particular, panels A and B (Fig. 4.11) report IL6 release, NO production and iNOS expression in co-cultures of microglia/astrocytes treated with 100 nM CGRP in combination with the cytokine mix and LPS (10 ng/ml) for 24 hours. As expected from previous data, LPS triggered a marked inflammatory response, which is inhibited by the addition of CGRP in the culture medium. On the contrary, I found that CGRP treatment of cytokine-stimulated cells did not exert inhibitory effects on NO/iNOS and IL6 levels.

In panels C and D, pure astrocytic cultures were exposed to the same kind of combination of stimuli and also in this case the addition of 100 nM CGRP did not exert effects on the upregulation of the markers of activation by cytokine. In this case, as said before, LPS stimulation is mainly used as a sensitive tool in order to exclude contaminations of even small amounts of microglial cells. In fact, in panels C and D, I did not observe the induction of the activated phenotype upon LPS stimulation, thus letting me consider pure the astrocytic cultures.

Finally, as it is possible to appreciate in panels E and F, CGRP had no effects on cytokine stimulation even in highly enriched microglia cultures, thus suggesting that, under this condition, the activation process is sustained by various mechanisms and that CGRP modulates only specific pathways leading to cytokine and NO release from microglia.

Similar results were obtained with AM in highly enriched microglia cultures (panels E and F). In particular, 100 nM AM were added to cell cultures in combination with the cytokine mix for 24 hours, thus making overlapping the effects of these two neuropeptides.

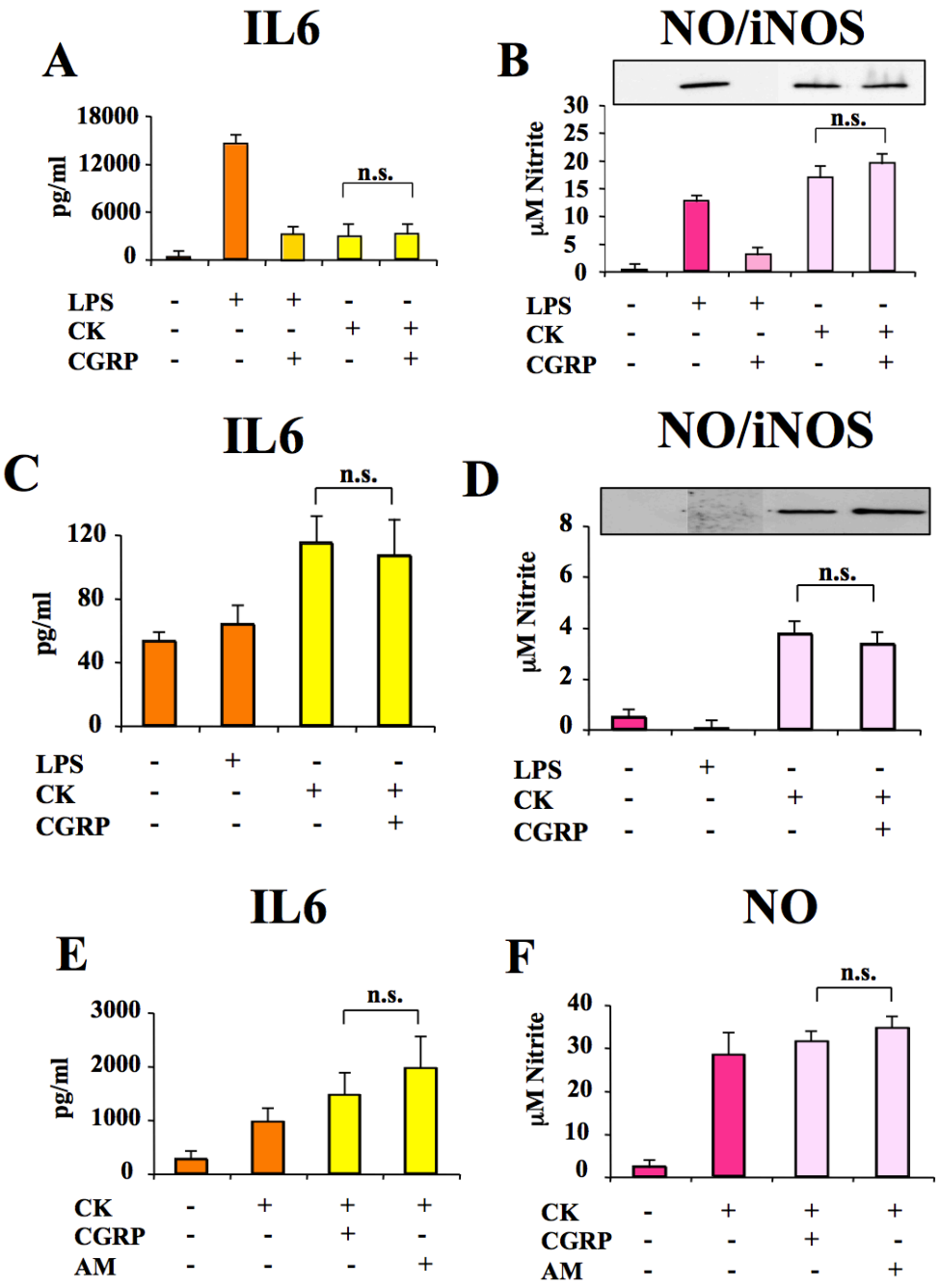


Fig. 4.11: The effect of pro-inflammatory cytokines is not modulated by CGRP or AM.
Microglia/astrocyte co-cultures (A and B), pure astrocytes (C and D), or highly enriched microglial cells (E and F) were stimulated by LPS (10 ng/ml) or a mix of cytokines (CK: 10 ng/ml IL1 β , 30 ng/ml TNF α and 20 ng/ml INF γ) for 24 h and the effect of CGRP (100 nM) or AM (100 nM) was tested on IL6 release (A, C, and E) and NO production/iNOS expression (B, D, and F). No statistical significant decrease (n.s.) was observed in the activation markers when CGRP or AM were applied to CK stimulated cells.

4.4.4 Inhibition of chemokine expression by CGRP and AM

Having established that CGRP and AM reduce microglial activation, I tested whether these same treatments were also able to affect chemokine production in LPS-stimulated co-cultures. Indeed, LPS promoted the transcription of some chemokines known to play a major role in neuroinflammation (e.g. CCL2/MCP1, CCL3/MIP1α, CXCL10/IP10 and IL10).

I stimulated microglia/astrocytes co-cultures with the protocol previously described (24 hours with 10 ng/ml LPS alone and in combination with 100 nM CGRP or AM), and then I performed a qRT-PCR. Of note, the two neuropeptides differentially affected the increases promoted by LPS treatment. CCL2/MCP1 and CCL3/MIP1α induction was strongly repressed by both neuropeptides: this is of interest since these two chemokines are implicated in the recruitment of T lymphocytes into the parenchyma of the central nervous system. CXCL10/IP10 increase was not significantly inhibited. On the other hand, the expression of IL10, which is potentiated in alternative pathways of microglia activation, was not increased by the two neuropeptides and even lowered by adrenomedullin. CGRP and AM were also ineffective in inducing genes related to the appearance of anti-inflammatory and neurotrophic phenotypes of microglia (Colton, 2009), such as Fc epsilon RII (CD23), transforming growth factor β, and nerve growth factor (see below Fig. 4.21).

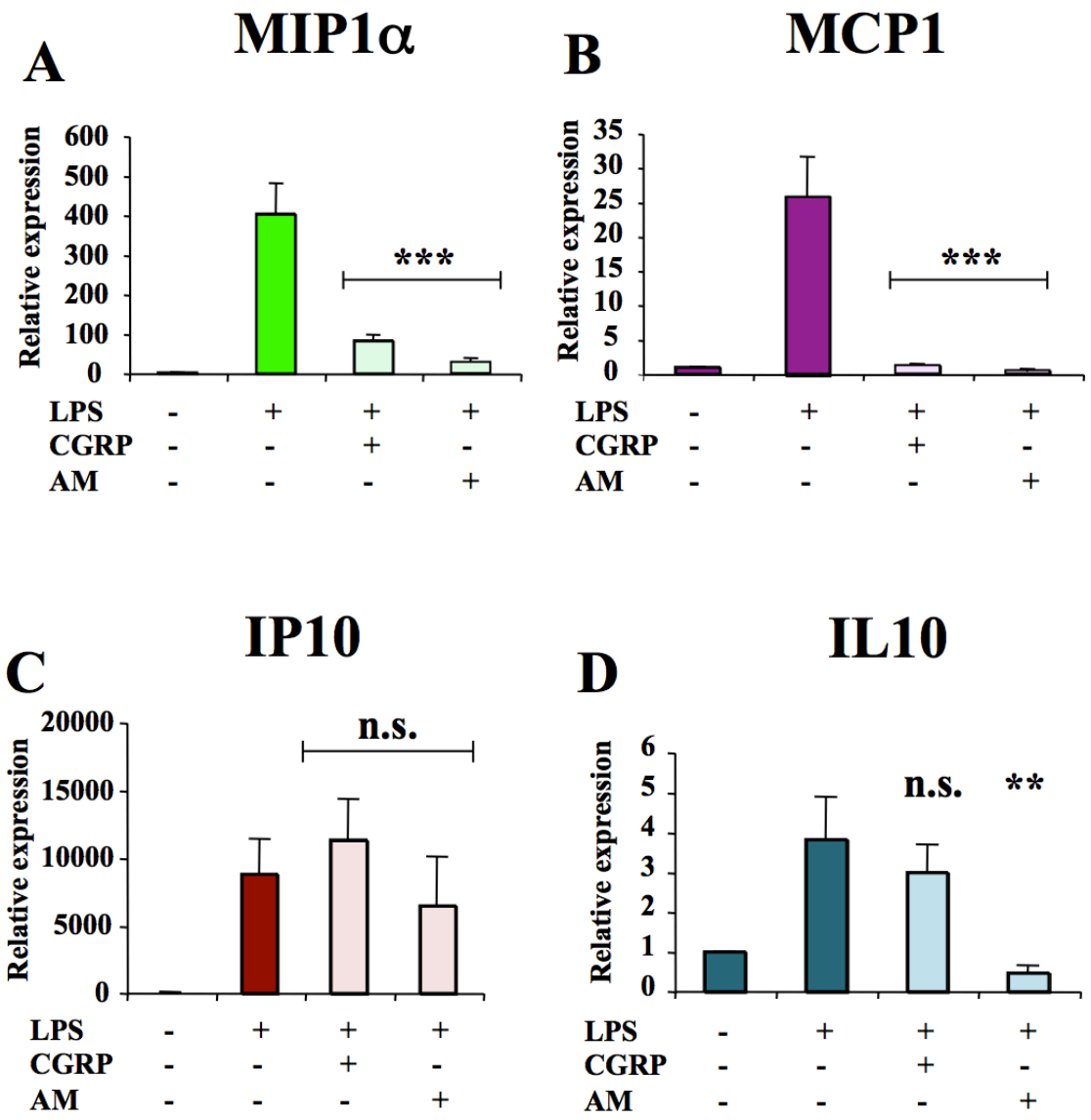


Fig. 4.12: CGRP and AM revert induction of chemokines by LPS.

Relative variations in the expression of MIP1α (A), MCP1 (B), IP10/CXCL10 (C) and IL10 (D) measured by RT-qPCR in microglia/astrocyte co-cultures. Statistical significance (n.s. $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$) was calculated using one-way ANOVA followed by Dunnett's post hoc test (against the LPS treatment).

4.4.5 Role of cAMP and cAMP-related stimuli in the modulation of microglia activation

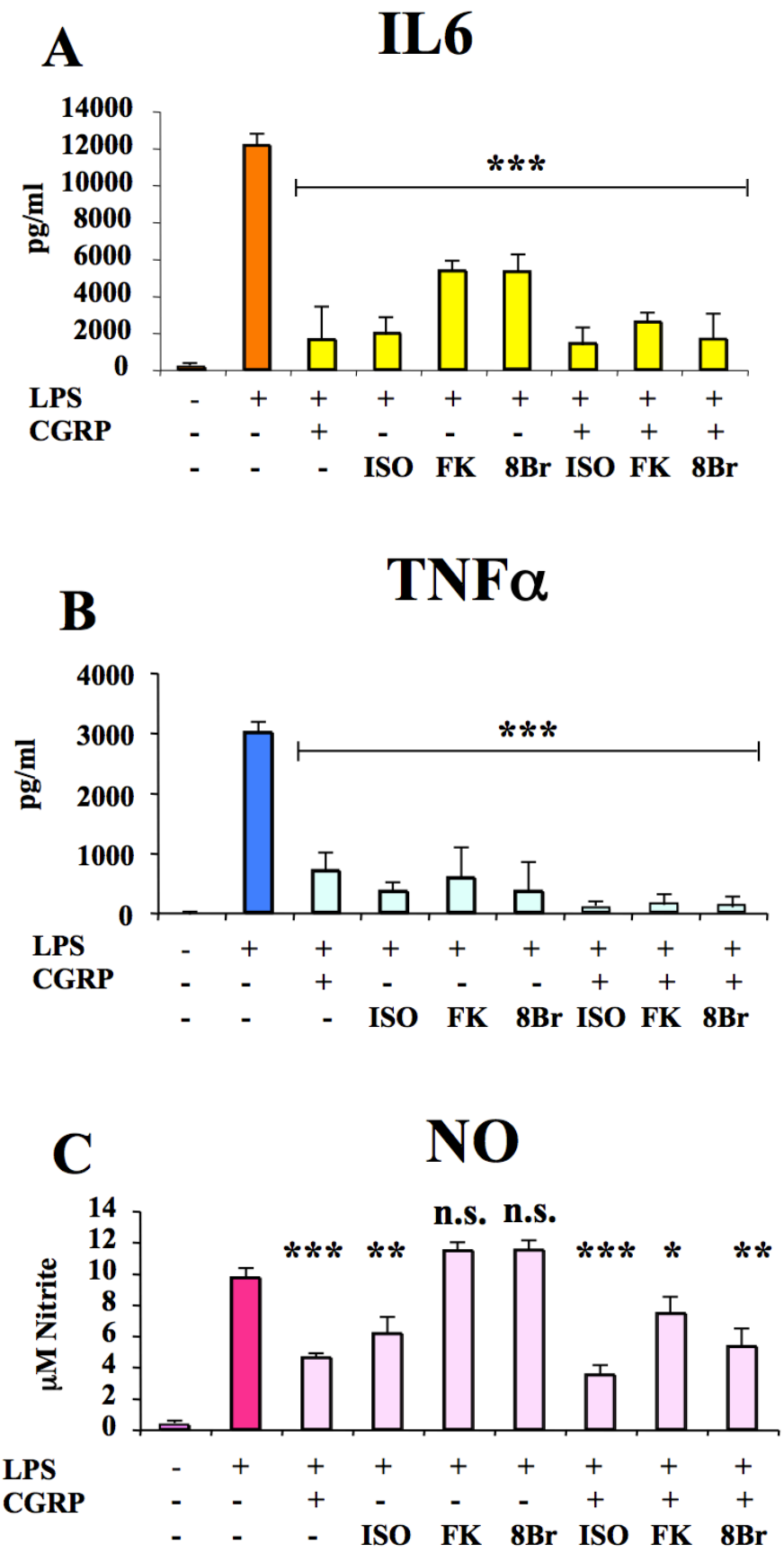
Since CGRP and AM are known to activate adenylyl cyclase and promote cAMP increase, I decided to expose cells to various treatments able to stimulate or mimic the cAMP pathway, in order to evaluate the involvement of this pathway in triggering CGRP and AM effects.

Stimuli used in this kind of experiment on microglia/astrocytes co-cultures were: isoproterenol, PGE_2, 8-Br-cAMP and forskolin, in combination or not with LPS and CGRP. Cells were preincubated for 30 minutes with 1 μM isoproterenol, 10 μM forskolin, 100 μM 8-Br-cAMP before LPS and CGRP stimulation.

As reported in panels from A to C (Fig. 4.13) isoproterenol, which is reported to activate β-adrenergic receptors on microglia (Tanaka. 2002), displayed the same effect of CGRP and AM, while other, non receptor-mediated protocols to elevate cAMP, had a more complex behavior. In fact, treatment with either 8-Br-cAMP, an analog of cAMP, or forskolin, an activator of adenylyl cyclase, prevented the LPS-induced increase in IL6 production and TNFα release, but not in NO elevation. Hence, receptor stimulation seems to elicit effects that go beyond mere cAMP increase.

Another stimulus commonly used to raise cAMP in microglia, PGE_2, was also effective in reducing LPS-mediated IL6 and TNFα release, but was not able to produce the same inhibitory effect on NO production (Panels from D to F). Rather, PGE_2 reinforced the LPS-mediated NO increase, most likely by stimulation of the prostaglandin E receptor 2 (EP_2), as revealed by the similar effects obtained with 1 μM butaprost (specific agonist for EP_2), but not 1 μM sulprostone (specific agonist for EP_3).

In light of the possibility that CGRP and AM may activate signaling pathways other than cAMP (Wang, 2009), I exposed LPS-activated cultures to PD98059 (50 μM) and SB203580 (10 μM), two specific inhibitors of the pathways linked to activation of mitogen activated protein kinases (MAPK). These drugs showed a strong inhibitory effect on LPS-induced microglia activation, but did not influence the effects of CGRP and AM (not shown).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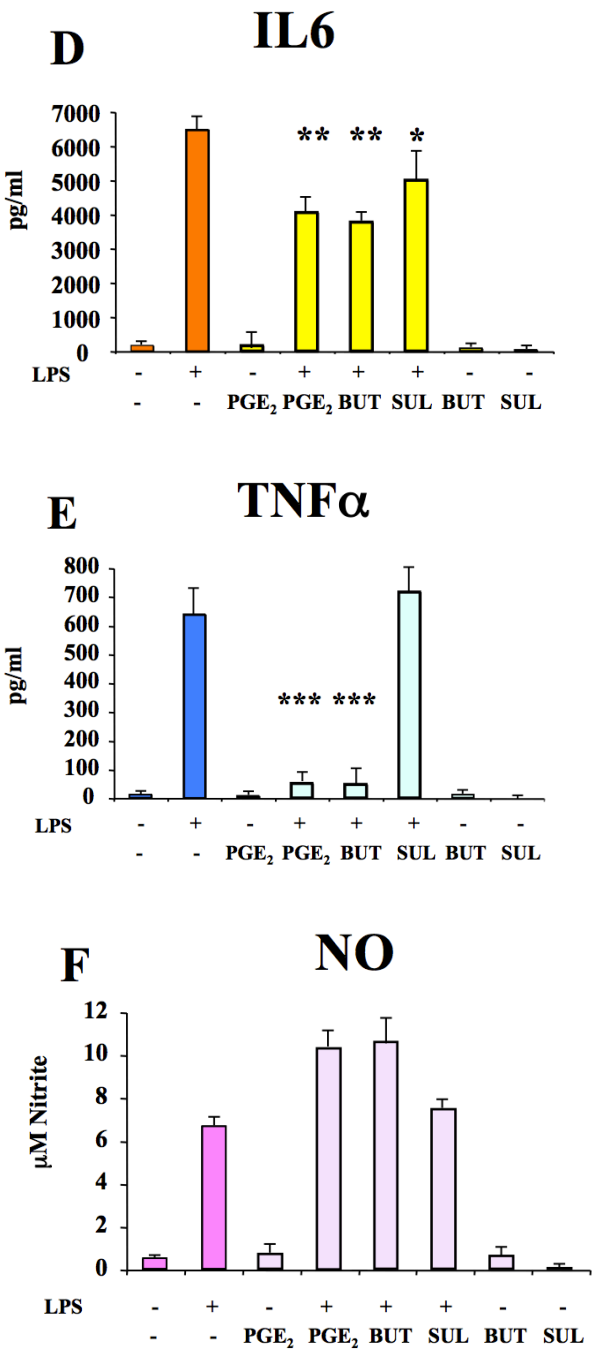

Fig. 4.13: Role of cAMP and cAMP-related stimuli on the modulation of microglia activation. Microglia/astrocyte co-cultures were stimulated with LPS (10 ng/ml) in the presence of various cAMP-modulating agents and the secretion of IL6 (A and D), TNFα (B and E), and NO (C and F) was measured. In addition to LPS and CGRP (100 nM), the other stimuli used were isoproterenol (ISO, 1 µM), forskolin (FK, 10 µM), 8Br-cAMP (8Br, 100 µM), PGE2 (1 µM), butaprost (BUT, 1 µM) and sulprostone (SUL, 1 µM). Statistical significance (n.s. $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$) was calculated using one-way ANOVA followed by Dunnett's post hoc test (against the LPS treatment).

4.5 Transcriptomic analysis of glia activation

In order to address the identification of the molecular mechanisms related to the activated phenotypes of glial cells, I performed a gene expression profiling approach in resting and activated primary cultures by exploiting the Illumina RatRef chip technology.

The gene expression profiling was analyzed on the following preparations.

1) Pure astrocytes treated 24 hours as described below:

- resting
- IL1β and TNFα (10 ng/ml, 30 ng/ml, respectively), with or without INFγ (20 ng/ml)
- conditioned medium from microglial cells activated with LPS (10 ng/ml)
- LPS (10 ng/ml)

2) Microglia/astrocytes co-cultures treated 24 hours as described below:

- resting
- LPS (10 ng/ml)
- LPS with fibrillar Aβ (5 mM)
- LPS with CGRP or AM (100 nM)

Total RNA from resting and activated cultures was analyzed for the expression of the ~22000 genes available on the chip.

The transcriptomic analysis offers the unique opportunity to evaluate the complex changes in expression that occur after glia activation. First of all, I used this approach to validate the results described in the previous chapters. Then, this approach represented a specific strategy to identify new genes related to glia activation and to recognize cell-type specific molecules differently expressed upon stimulation with either pro-inflammatory mediators or inhibitors of the activated phenotype.

4.5.1 Modulation of gene expression in pure astrocytic cultures

The initial step of the analysis of the transcriptomic data was the confirmation of the production of pro-inflammatory mediators during the various kinds of stimulation. First of all, I used pure astrocytic cultures to validate the in vitro model of activation. I treated cells with IL1β and TNFα (with or without the addition of INFγ), the conditioned medium from microglial cells activated with LPS (MCM^+), i.e. the condition that is closer to an in vivo situation, and LPS, as a negative control for the presence of microglial contaminants.

As illustrated in Fig. 4.14 expression of IL6, COX_2, MCP1 and MIP1α (panels A-C-D-E), was up-regulated with all the treatments (apart from LPS, indicating the correct purity of the preparation), while, as expected, iNOS expression was strongly induced by INFγ addition (panel B). The effects of the MCM^+ treatment were similar to the stimulation with IL1β and TNFα, suggesting that these two cytokines, and not INFγ, were released from activated microglia. However, a careful analysis of the data revealed important differences between the stimulation with the two cytokines and that with conditioned medium (see Fig. 4.15)

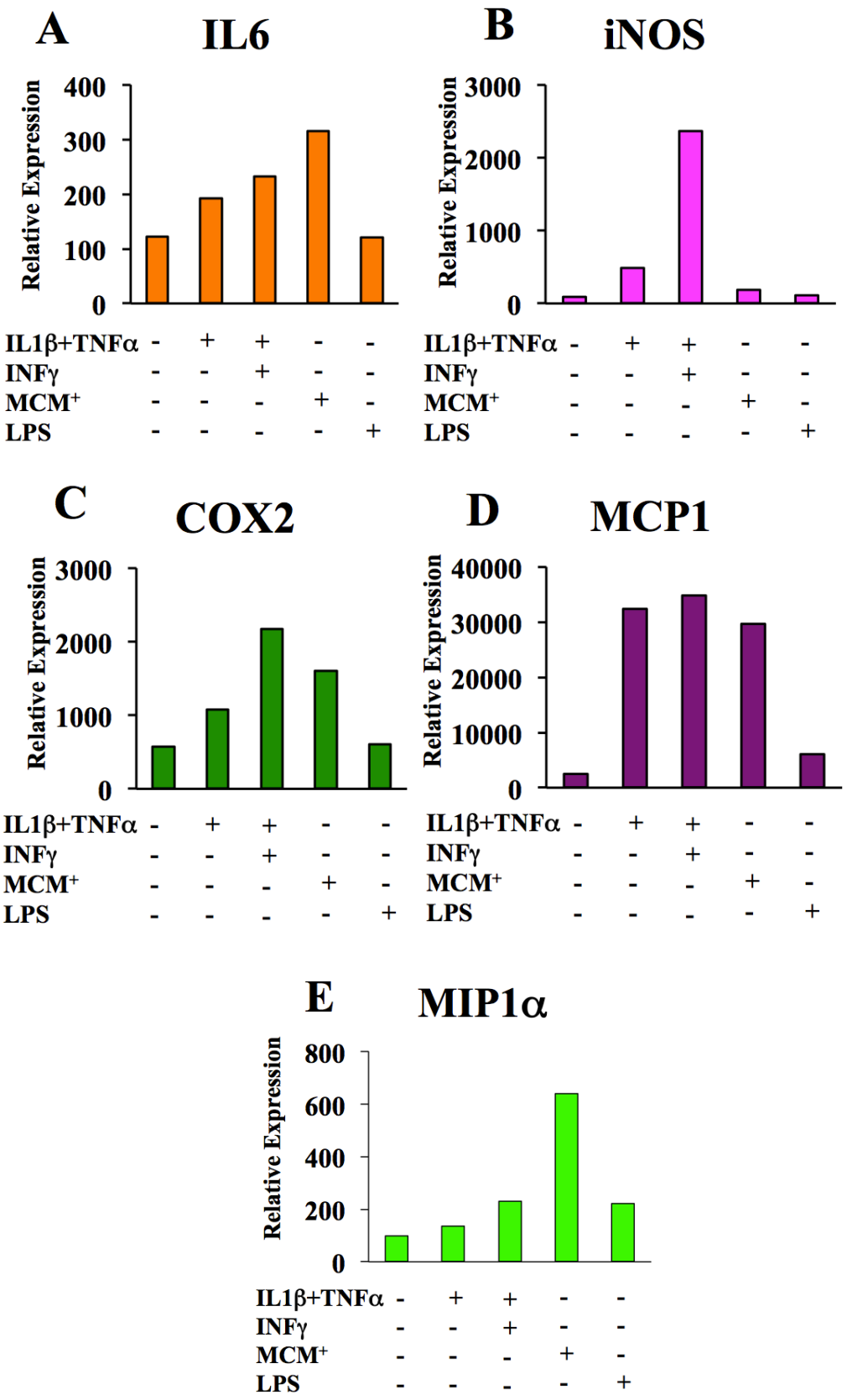

Fig. 4.14 Validation of in vitro astrocyte activation by transcriptomic analysis

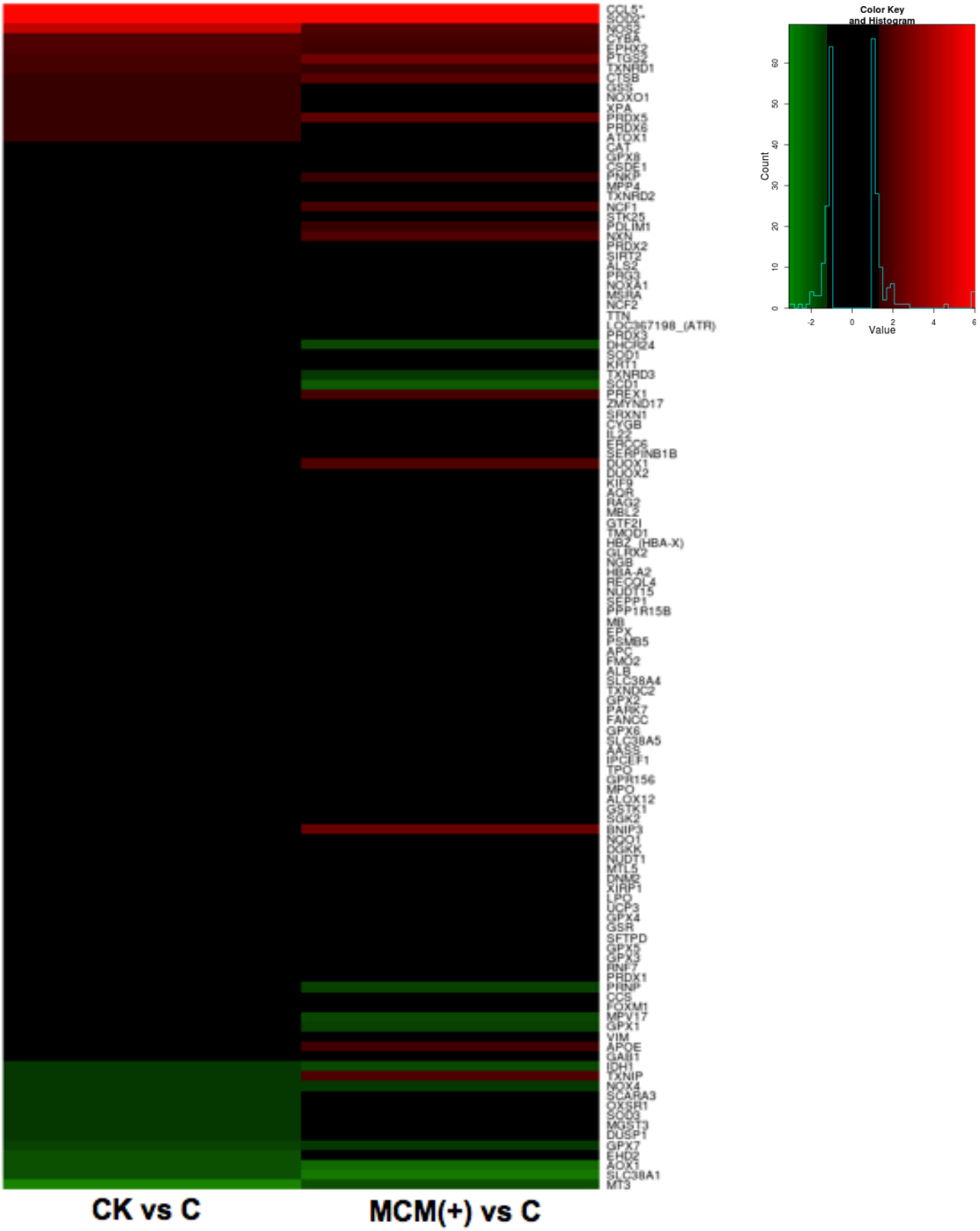

Fig. 4.15: Heatmap of upregulated (red) and downregulated (green) genes (the threshold with respect to the control condition was set at 1.3 fold listed in descending order according to the IL1β and TNFα stimulation.

4.5.2 Effects of fibrillar amyloid β on gene expression

In order to investigate at the molecular level the mechanism of amplification of LPS effects mediated by fibrillar Aβ at the molecular level, I started to analyze in astrocytes/microglia co-cultures the expression of the classical pro-inflammatory mediators produced during activation (i.e IL6, COX_2, iNOS, MCP1, MIP1α, IL1β).
As expected, all these markers of inflammation were found to be up-regulated by LPS stimulation, and this increase was amplified when LPS was administered in combination with fibrillar Aβ (Fig.4.16).

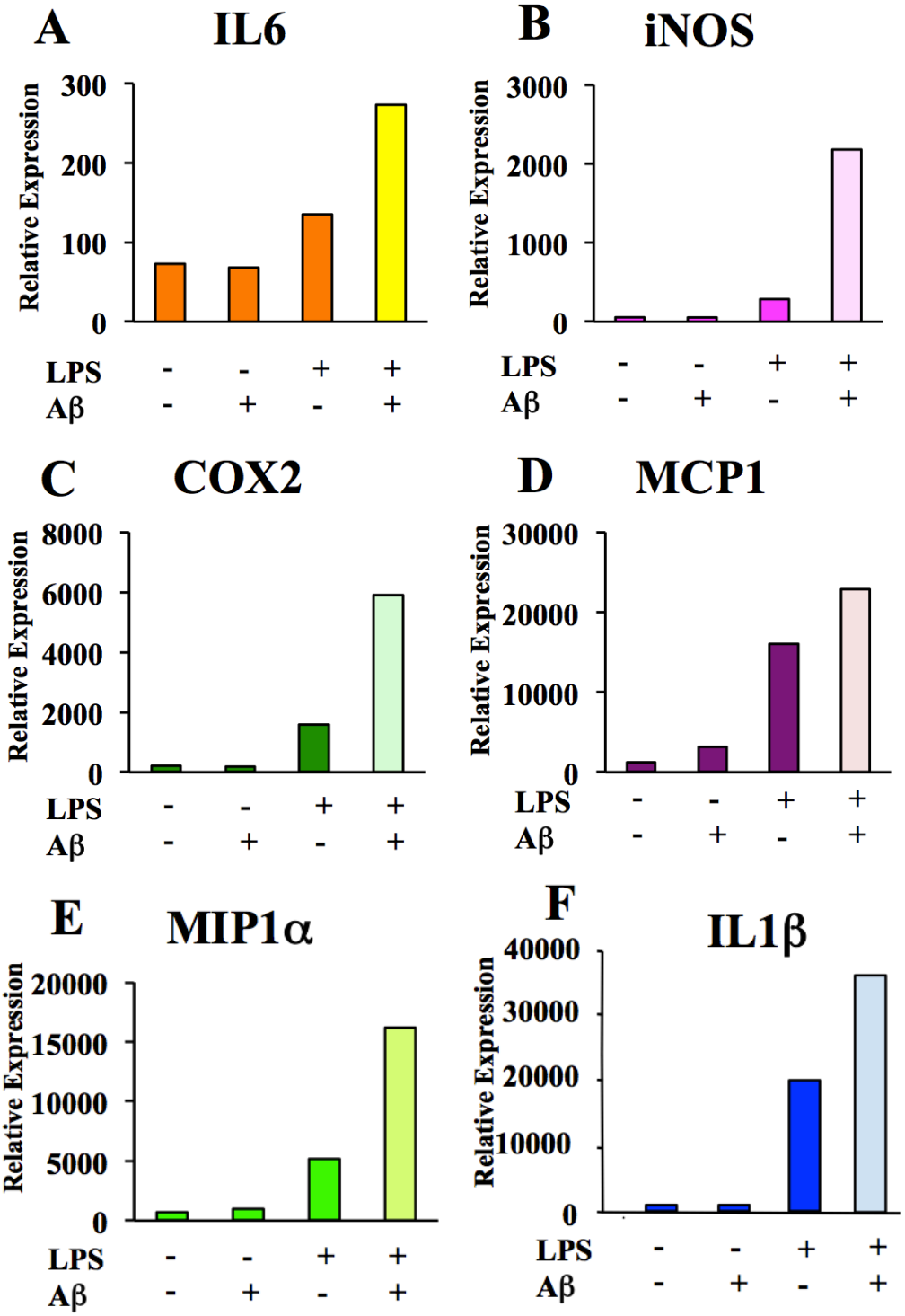

Fig. 4.16 Validation of the increase of the LPS effects mediated by amyloid β by transcriptomic analysis

It was once more evident that this amplification could not be explained by a simple additive effect since fibrillar Aβ alone was found to be almost ineffective, as illustrated in Fig. 4.17. In this analysis it is possible to appreciate the noteworthy number of genes that are differentially expressed upon LPS treatment (Panel A), while, in panel B, it is possible to observe the minor changes of gene expression levels upon stimulation with Aβ.

This observation opened the possibility that the addition of fibrillar Aβ to LPS stimulation was able to change the activation response, not only in quantitative, but also in qualitative terms. This aspect was further analyzed by comparing the patterns of gene expression obtained with LPS alone or in combination with Aβ (Fig. 4.18). It is evident from the graph that there are important qualitative differences in the expression of genes modulated by LPS with or without Aβ administration, thus highlighting a possible contribution of Aβ in triggering a distinct microglia activated phenotype, rather than merely potentiating of LPS effects. This kind of analysis will be extremely useful to identify biochemical markers that characterize the Aβ–mediated response.

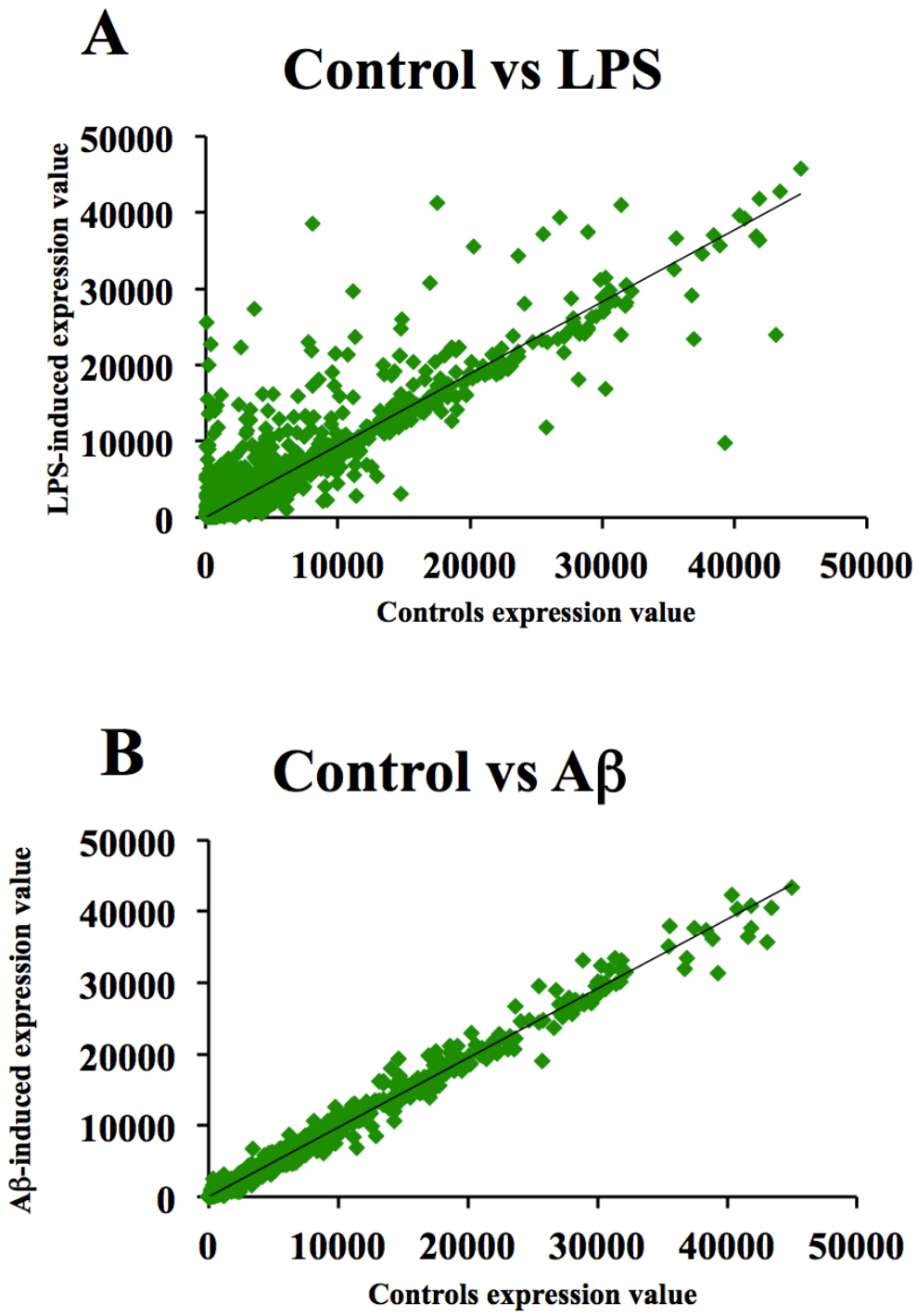

Fig. 4.17: Gene expression modulation during stimulation

Graphical representation of genes up- or down-regulated upon stimulation with LPS (A) and Aβ (B). LPS promotes a modulation of the expression of various genes while fibrillar Aβ alone was almost ineffective

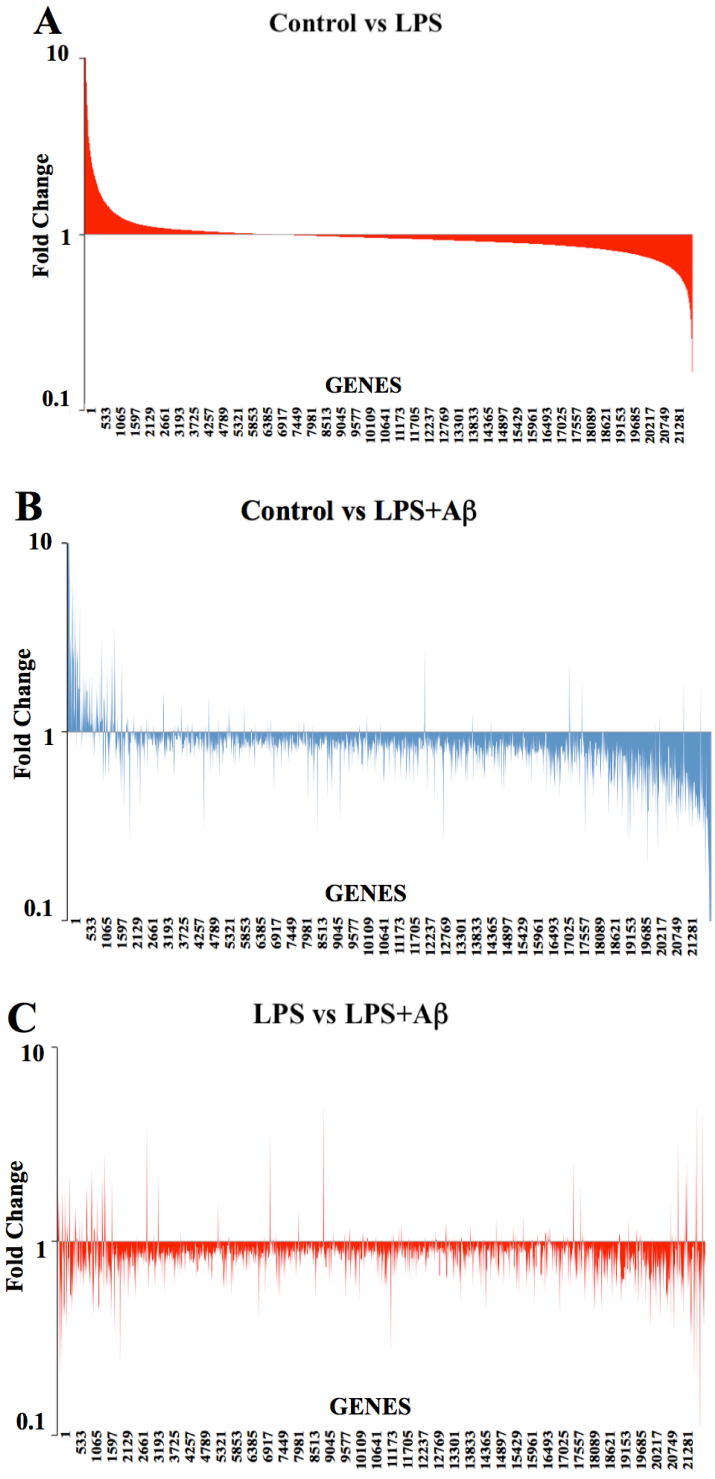

Fig. 4.18: Modulation of gene expression during LPS and Aβ stimulation

Graphical visualization of genes up- or down-regulated upon stimulation with LPS (A) and LPS+Aβ (B) Peaks in graph C represent genes that are differentially expressed when fibrillar Aβ is administered in concomitance with LPS.

4.5.3 Effects of neuropeptides on LPS treatment

In a second set of analysis, I validated the inhibitory effects of CGRP and AM on LPS stimulation. As expected from the biochemical results, I observed a decrease in all the pro-inflammatory molecules used as markers of activation (i.e IL6, COX_2, iNOS, MCP1, MIP1α, IL1β), also from the Illumina data, as represented in Fig.4.19.

More relevant, as illustrated in Fig. 4.20, the expression profile of a sample group of chemokines suggested that the effects of CGRP and AM are mainly addressed to inhibit of LPS stimulation. As described in the introduction chapters, microglia can change its phenotype in relation to various stimuli, thus acquiring different activated states. However, from the analysis of the expression of markers related to the "alternative activation" of microglia (Colton, 2009), it is clear that the effect mediated by the neuropeptides can be attributed to a maintenance or reversion to the "resting" state. As represented in Fig. 4.21, in fact, administration of CGRP or AM in association with LPS, did not change the expression of FCεRII, NGF, TGFβ and Ym1 genes, recently proposed as markers of the alternative activation pathway (Colton, 2009).

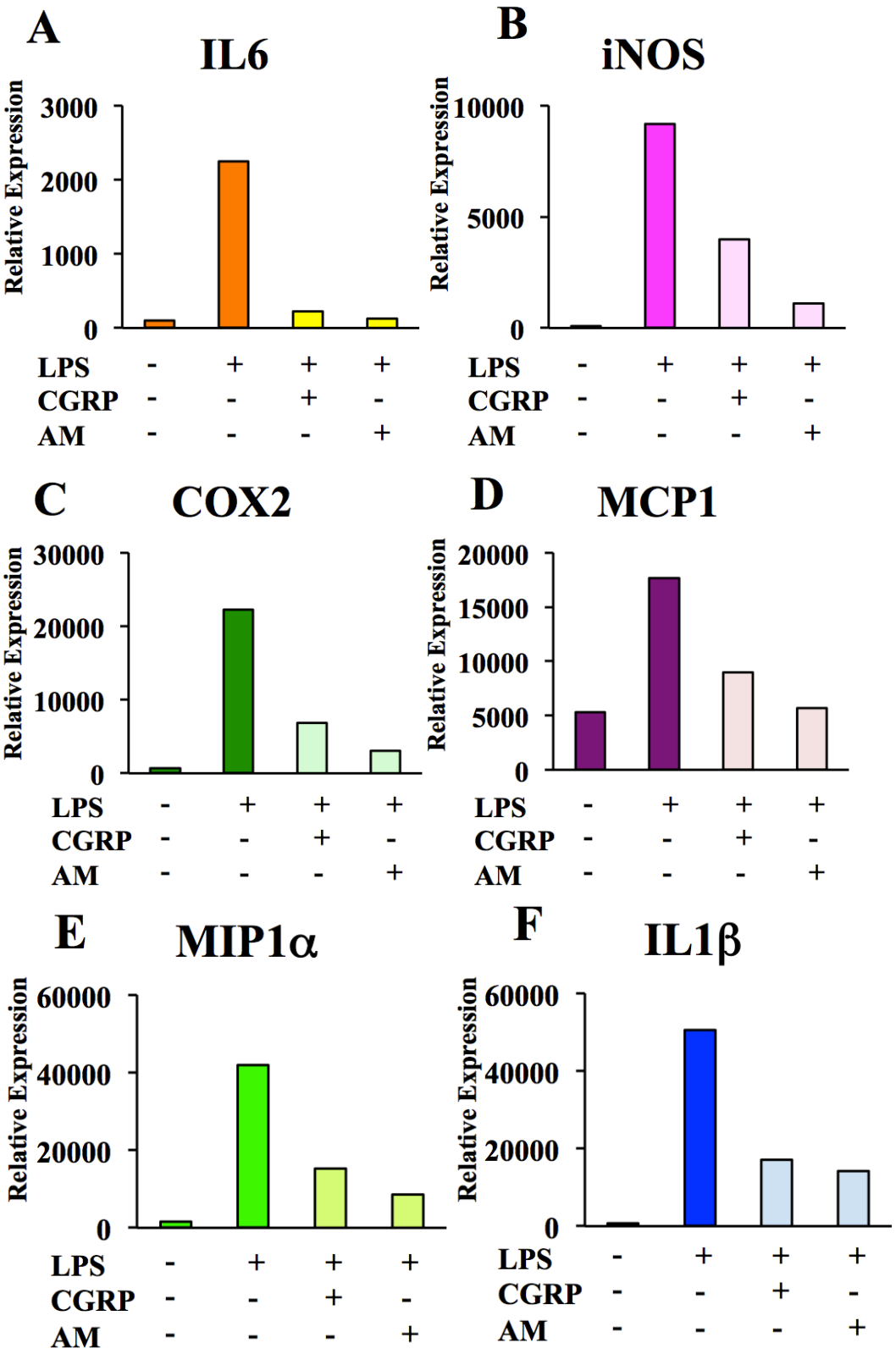

Fig. 4.19 Validation by transcriptomic analysis of the inhibition of LPS effect mediated by CGRP/AM

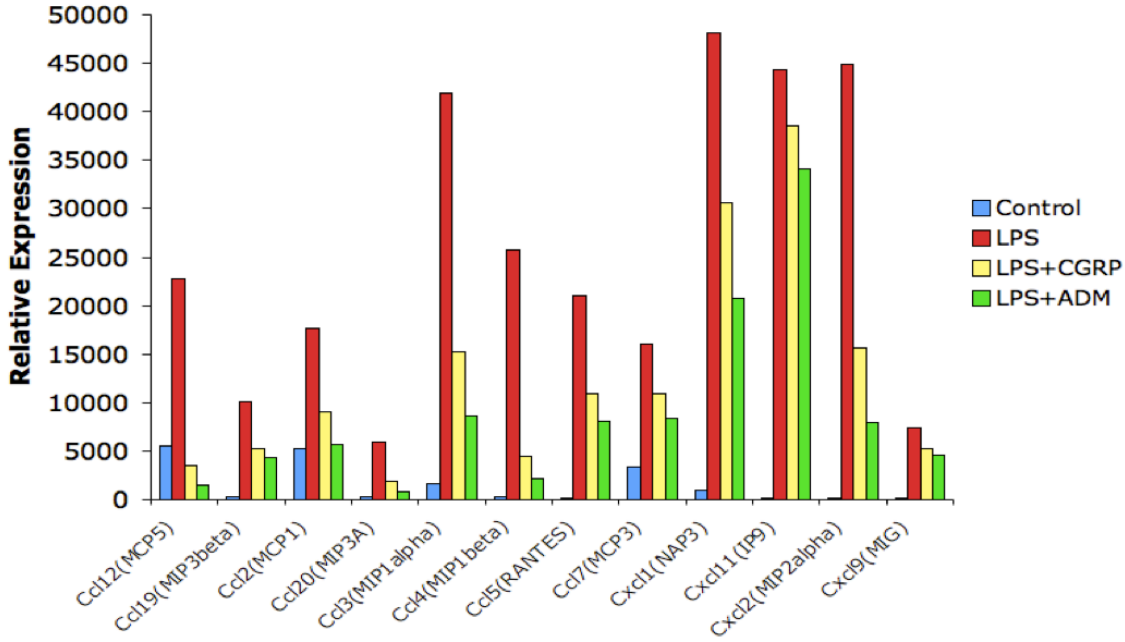

Fig. 4.20 Chemokine expression profile upon LPS stimulation in the presence of the neuropeptides

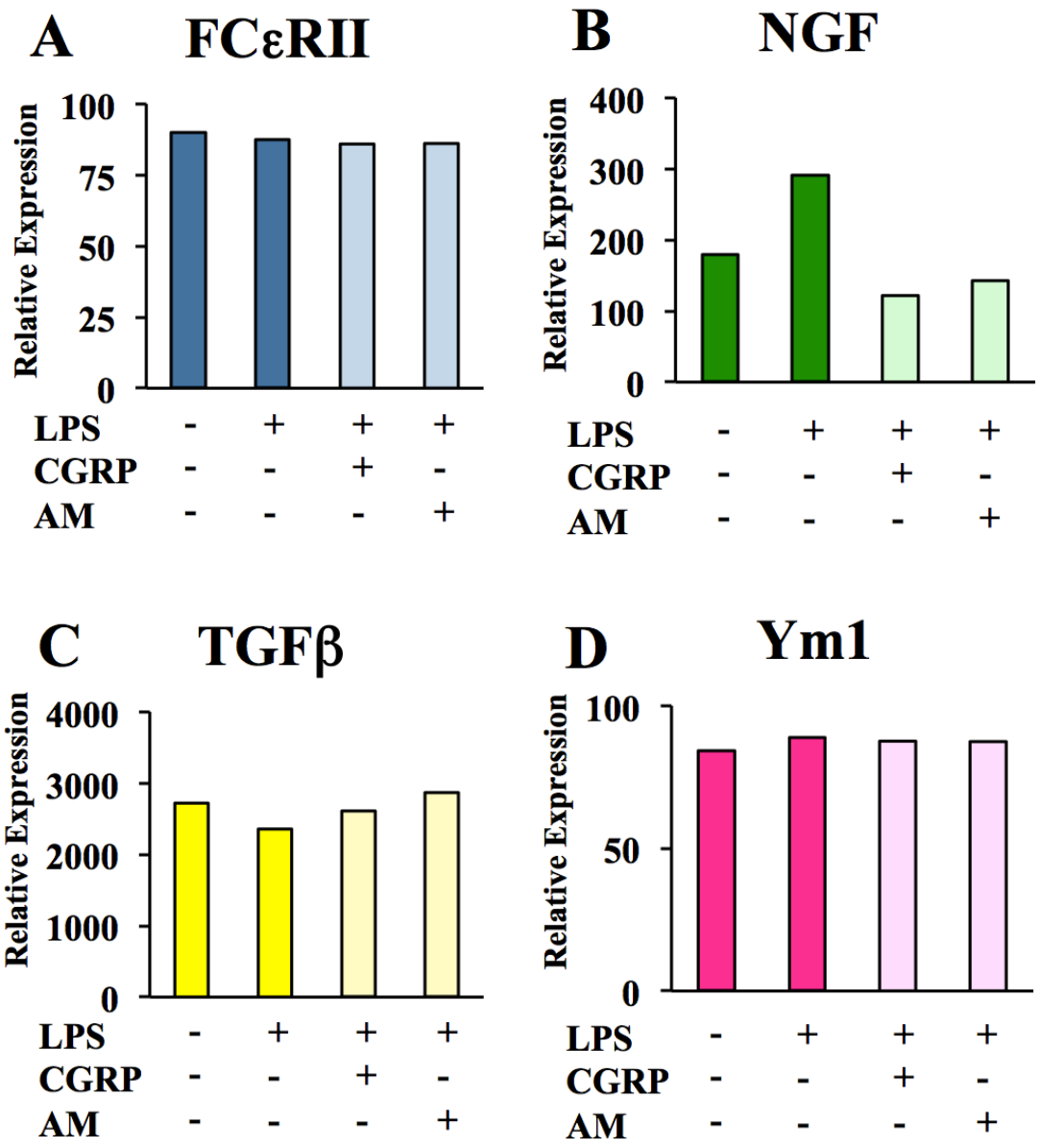

Fig. 4.21: Modulation of markers of microglia alternative activation

CGRP and AM are not able to induce up-regulation of "alternative activation" markers that are described in the literature. Inhibition of LPS effects induced by these two neuropeptides seems to revert microglia phenotype to a resting state.

5. DISCUSSION

Neuroinflammation is an essential element of a range of neurodegenerative diseases and their associated neuropathological features. Increasing evidence suggests that glial cells and their activation are key factors in this process. In particular, acute glia activation is a process occurring frequently in the CNS in response to physiological or pathological signals, while chronic activation is one of the features of brain injury and neurodegenerative/neuroinflammatory diseases.

The conversion of glia from the resting to the reactive state has been characterized in animal models of acute CNS injury and neurodegeneration. However, in the in vivo condition, it is not easy to investigate the molecular mechanisms of the glial pathology, because of the complex integration of responses of the many cell types present in the CNS, including neurons, glia, and, in certain cases, infiltrating leukocytes. For this reason the first aim of my project was to reproduce the process of reactive gliosis in an in vitro model, starting from the setting up of the conditions for controlled cell culture preparations.

Primary glial cultures can be obtained from virtually any CNS region and, if carefully prepared, are reproducible in their responses and versatile. One important aspect of my work was to highlight the effects of the presence of microglia in glial cultures, since it is known that that specific stimuli can directly act on microglia cells inducing the release of pro-inflammatory mediators that can, in turn, activate astrocytes. Activation leads to the modulation of gene expression in astrocytes, with increases in growth factors, cytokines, pro-inflammatory enzymes, cytoskeletal proteins. Therefore, astrocytes can contribute to restore the homeostasis by releasing signals that function as a feedback on microglia activation or, alternatively, they can contribute to amplify the inflammatory context.

In the literature the issue of the role of microglial cells in glial cultures has been recently highlighted (Saura, 2007). In fact, it has been proposed that “even small amounts of microglial cells can be responsible for effects observed on cultures in which the astrocyte is the most abundant cell type, thus making difficult the comprehension of the real activated phenotype” (Saura, 2007).

In this context, my first technical goal was to obtained cell cultures with a very low

level of basal activation without interfering with their proliferation state. In the meantime maximal effort was exerted to minimize or keep under control the amount of microglia. To these purposes, I set up growth conditions with horse serum in the culture medium, instead of fetal calf serum, which is largely used in literature. Moreover I optimized the shaking protocol commonly used to remove microglia, in order to obtain astrocytes either pure or with a controlled amount of microglia. By this optimization, I could obtain pure astrocytic cultures, highly enriched microglial cultures, co-cultures with a controlled relative percentage of cells (i.e astrocytic monolayers with a specified percentage of microglial cells), and astrocyte/microglia co-cultures with an approximate 1:1 ratio.

In order to study the process of glia activation on these cultures, I set up the activating conditions by using cytokines, i.e IL1β, TNFα and INFγ, as well as LPS, which are largely used in the literature as pro-inflammatory agents. Moreover, LPS treatment could also be used as a sensitive tool to detect microglial contaminants in astrocytic cultures. The activated phenotype was easily verified through the detection of secreted molecules (i.e IL6, PGE_2, NO, TNFα) or up-regulated proteins (i.e. iNOS, COX_2).

With the cellular models under control and the right conditions to reproduce the activated context, I could move on to study the modulation of the activation process by testing the effects of the various stimuli that might act either directly on cells or by producing indirect inflammatory effects through LPS and cytokines. This primary culture approach also represents a useful tool also to study the crosstalk between astrocytes and microglia in both the resting and the activated state. Moreover, it emphasizes the importance of the in vitro assays to test molecules possibly involved in the modulation of complex physiological and pathological processes, such as glia activation.

5.1 A new type of Aβ-mediated activation of glia

The evidence that the deposition of the Aβ peptide in the brain parenchyma is the common feature of all forms of AD has represented a fundamental step for the investigation of the molecular basis of the AD pathogenesis, giving rise to the “amyloid

hypothesis", i.e. the assumption that Aβ aggregation is the key event leading to neuronal impairment and neurodegeneration. This hypothesis includes the importance of the chronic inflammatory context that is originated by the interaction of Aβ with glial cells. In fact, apart from direct neuronal damage, Aβ aggregates have also been reported to promote glia activation with the concomitant release of pro-inflammatory cytokines, chemokines, NO and ROS, which may contribute to neuronal death (Kitazawa, 2004). In particular, this inflammatory response is mediated by local glial cells without any apparent contribution by immune cells from the periphery. Although the initiators of inflammation in AD remain unclear, activation of microglia appears to occur at a very early stage of AD. In addition to microglial activation, reactive astrocytes have also been strongly associated with the pathology of AD. These activated microglia and reactive astrocytes are found to be intimately associated with the neuritic plaques and these cells surround amyloid aggregates with dense layers of thick processes.

While it is widely accepted that accumulation and aggregation of Aβ may promote synaptic alterations, glial activation and progressive neuronal loss, the mechanisms at the basis of these pathological changes are largely unclear, not only in terms of the cellular target of the Aβ action, but also in terms of the molecular forms of Aβ and their corresponding receptors. Since oligomeric forms of Aβ can directly cause synaptic dysfunction and loss, as well as long-term potentiation disruption (Deshpande, 2006, Walsh, 2002) they have been proposed as the most pathological Aβ form. From the molecular point of view, this Aβ form could target neurons, inducing variations in their ionic homeostasis, with excessive calcium entry that would contribute to neuronal dysfunction and death.

Alternatively, evidence in the literature proposes that also fibrillar Aβ can have direct toxic effects on neuronal cells by changing the permeability of plasma membrane through the interaction with the lipid bilayer, and also by interfering with axonal and dendritic transport (Lazarov, 2007, Smith, 2007, 2005). Other possible mechanisms proposed to be involved in Aβ toxicity on neurons are the induction of the apoptotic cascade (Loo, 1991) and mitochondrial dysfunction (Hirai, 2001, Manczak, 2006).

In my work I used the model of glial cultures to test in vitro the effects that the various forms of Aβ can have on glia. In fact, in the literature, it has been shown that Aβ (both oligomeric and fibrillar) induces high levels of iNOS expression and NO

release in pure rat cortical astrocytes (White, 2005). For doing this investigation, I first addressed the issue of the protocols commonly used to obtain monomers, oligomers and fibrils.

I solubilized the peptide in dimethylsulphoxide (DMSO) to obtain monomers, while oligomers and fibrils were prepared by maintaining an Aβ solution in red phenol-free EMEM for 24 h at 4°C and one week at 37°C, respectively. HCl solubilization of Aβ was not employed since the solvent interfered with the glia activation process. Unexpectedly, I found that Aβ alone, in any aggregation state tested, is not able to activate astrocytes or to induce glia activation even when microglia is present in the cultures. However, I observed that fibrillar Aβ induces a potentiation of the effects of LPS and cytokines on co-cultures, thus highlighting the relevance, for the Aβ effects, of the inflammatory context created by astrocytes, microglia and, possibly, other players through the release of biological mediators.

I named "superactivation"this effect of Aβ, in order to emphasize the action of amplification of activator stimuli by Aβ. This result become important if integrated in the context of the interplay between the two types of glial cells analyzed in this thesis, with particular regards to the presence of specific receptors for fibrillar Aβ, as well as Aβ-activated transduction pathways that should be better characterized in the near future, especially in view of possible anti-inflammatory approaches aimed to address Aβ toxicity.

5.2 Neuropeptides and glia activation: a new feedback mechanism?

The inhibitory effects of CGRP and AM that I reported in my work were clearly stimulus dependent and cell specific. In fact, while CGRP and AM alone were almost ineffective, they were effective and potent in inhibiting LPS activation of microglia.

By adopting the previously described model of in vitro glia activation, I demonstrated that CGRP and AM efficiently inhibit the LPS-mediated effects. Accordingly, my data pointed to microglia as the primary target of this cascade of events, while astrocytes seemed to be involved because activated through molecules released by microglia. It is worth mentioning that, according to the current view on the neuroinflammatory process during neurodegeneration, microglia is considered the

causal player, with astrocytes exerting either a protective feedback role, by limiting microglial activation (Harris, 2002), or a deleterious feed-forward effect, by favoring the development of chronic inflammation (Farina, 2007).

Notwithstanding the extent of interplay between microglia and astrocytes, which is far from being elucidated, it is clear that CGRP and AM can play an important role on the effects of microglia activation. These two neuropeptides reverse LPS effects rather than redirect microglia to different pathways of activation, such as the anti-inflammatory "alternative activation" or the immunosuppressant "acquired deactivation" phenotypes proposed in the literature (Colton, 2009). This data is confirmed by the transcriptomic analysis, in which stimulation with CGRP and AM did not induce an up-regulation of the specific markers for additional microglial phenotypes. Moreover, it should be pointed out that CGRP and AM inhibition on microglia activation is exerted on specific pathways. In fact, I did not observe the inhibitory effect when cells, either astrocytes or microglia, were challenged with a mix of pro-inflammatory cytokines, instead of LPS.

Since both neuropeptides activate the CLR complexes, it is possible that a common signal transduction pathway may sustain their effects. Indeed, cAMP is produced upon stimulation of CGRP or AM receptors and is thus expected to be involved in the inhibitory activity observed on LPS action. However, elevation of cAMP levels by common pharmacological tools mimicked the effect only partially, thereby suggesting, as already reported (Walker, 2010), that receptor activation stimulates additional signaling pathways. Interestingly, when I stimulated other receptors coupled to adenylyl cyclase I obtained similar, as well as specific effects.

For instance, activation of β-adrenergic receptors mimicked the action of the two neuropeptides, while activation of EP2 receptors had an opposite effect regarding NO production. Altogether, the heterogeneous responses I observed are in line with the possibility that receptors coupled to adenylyl cyclase activation can stimulate, along with cAMP production, other signaling pathways as well, thereby driving glia to specific activated phenotypes.

Taken together, my results highlight the anti-inflammatory activity of CGRP and AM, thus opening new perspectives in the pharmacological treatment of pathologies characterized by microglia activation and neuroinflammation.

In this respect, multiple sclerosis is a paradigm, since all demyelinating lesions occur on an inflammatory background that implicate the involvement of several cells, including activated microglia and locally recruited lymphocytes and macrophages (Lassmann, 2001). The mechanisms leading to this pathological pattern are complex and poorly understood.

It has been hypothesized that unknown inflammatory events may cause a local release of inflammatory cytokines (TNFα, IL1β, and IL6) capable of triggering the production of secondary inflammatory mediators, such as chemokines, colony stimulating factors and lipid-derived molecules (Martino and Hartung, 1999). Cytokines and chemokines would, in turn, lead to the recruitment of leukocytes across the BBB, including autoreactive T cells able to interact with resident antigen-presenting cells (Engelhardt and Ransohoff, 2005), such as microglia and astrocytes.

This local interplay is considered a crucial event that leads to perpetuation and amplification of the inflammatory reaction, since the initial damage introduced by T cells is a stimulus for microglia activation and further recruitment of macrophages.

Within this pathological context, activated microglia and infiltrating macrophages are expected to produce a large number of harmful soluble factors, such as nitric oxide (Redford, 1997), inflammatory cytokines or chemokines (Muzio, 2007), excitotoxins (Smith, 2000), matrix metalloproteinases (Leppert, 2001; Lindberg, 2004) and other proteases (Anthony, 1998). However, a view in which microglia might exert a reparative and anti-inflammatory action, with mechanisms still to be clarified, has recently emerged.

In this context, the immune activity of CGRP and AM might exert either a control on the initial immunological attack to the CNS parenchyma, but also on subsequent mechanisms of the disease, via the modulation of glia activation. This is supported by recent studies in which changes in CGRP and AM levels were observed in the EAE mouse model of relapsing–remitting multiple sclerosis (Morara et al., personal communication).

Noticeably, among the CGRP-induced effects on the CNS, its role in the development of neuropathic pain and migraine has attracted most of the attention (Recober and Russo, 2009). In fact, CGRP inhibitors are under evaluation for the therapy of migraine (Durham and Vause, 2010). In light of a possible physiological role

of CGRP in keeping neuroinflammation at bay, treatments with those inhibitors might have previously unpredicted harmful effects. On the other hand, this development of non-peptidic antagonists for the CGRP receptor (Doods, 2000), paves the way to the discovery of non-peptidic agonists for the same receptor family (Katayama, 2001), which might be employed in the treatment of neuroinflammatory diseases.

5.3 Future perspectives of the in vitro transcriptomic analysis

The transcriptomic analysis of genes modulated during various stimulating conditions opens a novel scenario regarding the possibility of identifying putative markers of microglia activation. Such molecules, differentially expressed upon stimulation with pathological agents, would be instrumental in the characterization of the microglial phenotype, giving rise to the possibility of obtaining new biomarkers that might be either disease-releated or specific for a defined pathological and cellular process. In addition, genes with a modified expression profile could be isolated and clustered according to their functions and to the cell type in which they are expressed. For instance, genes expressed at very low level in resting microglia, highly induced in the inflammatory condition (i.e. LPS stimulation in association or not with pathological stimuli including Aβ) and expressed at very low level in both resting and activated astrocytes, could be investigated as specific markers for activated microglial cells.

Clusters of genes could also be recognized and analyzed according to a pathway analysis, and the validation of putative genes of interest could be performed in vitro and ex vivo (e.g. by western blot, immunofluorescences on cultured cells, ELISA assays on biological fluids and immunohistochemistry on organotypic brain cultures and biological samples). If developed, disease-specific biomarkers in CSF or blood could play an important role in preclinical diagnosis, in order to promote early therapeutic intervention, as well as tools to predict the disease progression or the response to drugs.

The impact of the identification and validation of these new biomarkers includes the improvement of the in vivo visualization techniques (PIB-PET, fMRI) and the development of disease-modifying therapies.

In this context, for example, the currently approved therapeutic options for AD have a limited impact on disease progression and no disease-modifying therapy is available.

Moreover, until now, diagnosis is mainly based on clinical assessment underlying the need of both the validation of the CSF biomarkers that has been proposed for routine clinical use (such as the Aβ 1-42 peptide, or the ratio between phosphorylated tau and total tau), and the identification of new biomarkers. These markers, in combination with the development of therapies targeted to the pathological mechanisms specific for each disease, may have a strong impact on health care by improving the efficiency of the clinical trials and by accelerating the evaluation and discovery of effective treatments for neuroinflammatory and neurodegenerative diseases.

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